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14. ABSTRACT Steroid receptor RNA activator (SRA) was shown to differ from all previously characterized co-activators as it was demonstrated to function as a RNA rather than a protein molecule. We have however demonstrated that this once thought non-coding RNA encodes a well conserved protein (SRAP). The aims of this year are mainly on identification of SRAP-interacting Proteins and how SRAP interacts with transcriptional regulators to modulates of transcription, as well as the respective impacts of SRA RNA and SRA protein on the ER signaling pathway. We have determined through protein arrays that SRAP is indeed able to directly interact with various transcription factors. Furthermore, we have established that SRAP is associated to chromatin in MCF-7 cells. We also examined the possible effect of SRAP recruitment on transcription using the potent GAL4-VP16 hybrid transcription activation system. We observed that SRA possesses a transcriptional repressive activity capable of inhibiting the GAL4-VP16 transcription activity. This SRAP transcriptional repressive potential is sensitive to trichostatin A (a HDAC inhibitor) treatment. And SRAP is able to co-immunoprecipitate HDAC activity. Meanwhile, we have also investigated the possible mechanism of intron-1 retention as a participating to the generation of coding and non coding SRA RNAs.

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Introduction

Steroid receptor RNA activator (SRA) was discovered in 1999 and was shown to differ from all previously characterized co-activators as it was demonstrated to function as a RNA rather than a protein molecule. We have however demonstrated that this once thought non-coding RNA encodes a well conserved protein (SRAP).

To investigate the possible implications of SRAP expression on breast cancer progression, we examined by Western blot analysis ER positive primary breast tumors from patients subsequently treated with Tamoxifen. Our results showed that patients whose primary tumors were positive for SRAP expression had a significant lower likelihood to die from recurrent disease than SRAP negative patients. These results prompt us to investigate the possible effects of SRAP on the estrogen receptor signaling pathway.

In the first year, Shilpa Chooniedass had shown Progesterone Receptor is differentially expressed in the cells over-expressing SRAP. She had successfully generated the plasmids expressing functional SRA RNA and protein independently. Through mass spectrometric analysis of proteins co-immunoprecipitated with SRAP, she had identified 69 possible SRAP interacting proteins. Functional gene annotation classification revealed that several of these SRAP interacting proteins are involved in the modulation of transcription.

This year's aims were to mainly to identify SRAP-interacting proteins and how SRAP interacts with transcriptional regulators and modulates of transcription, as well as the respective impacts of SRA RNA and SRA protein on the ER signaling pathway.

Therefore, in this year, she has determined trough protein arrays that SRAP is indeed able to directly interact with various transcription factors. Furthermore, she has established that SRAP is associated to chromatin in MCF-7 cells. In light of these observations, we examined the possible effect of SRAP recruitment on transcription using the potent GAL4-VP16 hybrid transcription activation system. She observed that SRA possesses a transcriptional repressive activity capable of inhibiting the GAL4-VP16 transcription activity. This SRAP transcriptional repressive potential is sensitive to trichostatin A (a HDAC inhibitor) treatment suggesting an involvement of HDACs in SRAP mechanism of action. In support of this hypothesis, she determined that SRAP is able to co-immunoprecipitate HDAC activity.

Meanwhile, we have recently reported intron-1 retention as a possible mechanism participating to the generation of coding and non coding SRA RNAs.(see appendix 4) In order to investigate the role of SRA intron-1 retention we used splice-switching-oligonucleotides to increase the level of endogenous SRA intron-1 retention in the T5 breast tumor cell line. Following experimental optimization, we achieved a fifty-percent conversion in the proportion of non-coding (intron-1 retained) to coding (intron-1 spliced) SRA transcripts. Using real-time PCR array technologies we found that specific changes in the expression of genes implicated in breast cancer progression and estrogenic

signaling are associated with increasing intron-1 retention. Of particular interest is the observed increase in Upa (involved in invasion) and ER-beta (involved in estrogen signaling) gene expressions. (see appendix 4)

Body

Task 1: To characterize the cellular and biological effects of SRA and SRAP in MCF-7 cells

a) **To analyze the expression of ER target genes PR and pS2 in MCF-7 cells stably over-expressing SRAP.**

In the last year's report we had already concluded that the estradiol mediated regulation of the two ER target genes studied (PR and pS2) was dissimilar in the SRAP-V5 over-expressing and control cells. In fact, while the estradiol mediated induction of the PR was evidently distinct between the SRAP over-expressing and control cell lines, no difference in pS2 induction was observed between the cells. These results had been published. (See appendix 1 for detailed experimental procedures as well as detailed observations and interpretations)

b) **To determine the effect of SRA expression on the growth rate invasive properties of the MCF-7 cells over-expressing SRAP.**

In the last year's report, we demonstrated that a stably transfected cell model is not the appropriate tool to investigate the effect of SRAP expression on proliferation because growth rates of the two control cell lines (not expressing transfected SRAP) are significantly different from one another and we thus proposed to conduct proliferation assays in inducible SRAP MCF-cell lines. But we also want to conduct proliferation assays and invasion assay on cells in task2. (see task2)

Task 2. To characterize the respective actions of SRA RNA and protein on ER activity

a) **To develop plasmids that express functional SRA RNA and protein independently.**

In the first funding year we have successfully generated the four constructs that will allow us to characterize the respective actions of SRA RNA and protein. These four constructs are:

1. **SRARNA** that contain the full SRA sequence but with the first two ATG codons mutated. The corresponding RNA can not be translated (as it does not contain the correct initiating methionine codons) but will be functional at the RNA level (presence of an intact core region).

2. **SRAPROT** contains the full SRA cDNA but with the two series of silent mutations SDM1 and SDM7 previously shown to abolish the SRA RNA co-activator function. The corresponding RNA can not be functional but can encode the wild type SRA protein.
3. **SRARNAPROT** contains the full wild type SRA sequence. The corresponding RNA is translatable and fully functional.
4. **SRANEG** contains the full SRA sequence but with the first two ATG codons mutated and with the two series of silent mutation SDM1 and SDM7. The corresponding RNA should be non-functional and non-translatable.

All four vectors have been sequenced to confirm the incorporation of the appropriate mutations.

b) To determine the respective functions of SRA RNA and protein on reporter systems.

In the passed year, these four plasmid constructs had already been co-transfected in Hela cells together with an ERα expression vector (obtained from Dr. Murphy), an ERE-luciferase reporter gene (from invitrogen) and a Renilla luciferase control vector.(to normalize the transfection efficiency). However we didn't see neither significant increase of ligand-dependent ER activity in the presence of SRARNA, nor significant decrease in the presence of SRAPRO. These either due to low transfection efficiency or ERE-luciferase reporter constructs does not sensitive to be regulated by ERα and co-regulators. To solve these problems, we got now the four different EREs(EREc38, EREc13, fos-1211 and PR1148 EREs)-luciferase reporter constructs previously shown to be differentially regulated by ERα and co-regulators.(kindly provided by Dr. Klinge). Furthermore, we are trying to introduce lentivirus transfection system to boost up the transcription efficiency and better normalize the transfection conditions. Different SRAs and ERα constructs will be integrated following PCR-amplification into pLenti6/v5-D-TOPO vector using the viraPower lentiviral Directional TOPO Expresson Kit(invitrogen). ERE-luciferase constructs will similarly be introduced into a promoter-less system using the ViraPower Promoterless Lentiviral Gateway kit. The resulting pLanti-constructs will be transfected into 293FT cells and titer of corresponding lentivirus stocks harvested from medium supernatant will be determined. After setting-up optimal conditions for ER construct expression and optimal MOIs for E2 and tamoxifen treatment, we will assess the effect of different SRA constructs on ERα activity. For Hela cells, reporter and ERα constructs will be co-transduced with SRAs. Luciferase activity will be normaized to the amount of expressed SRA RNA transcript as assessed by real-time PCR. Four experiments will be performed and the different points of luciferase activity will be compared using the student's t test.

In addition, we are also going to try respective functions of SRA RNA and protein on ERβ activity by reporter gene assay because we found SRAP might has higher affinity to interact with ERβ.(see appendix 3 Table VII)

c) To determine the respective functions of SRA RNA and protein on ER target genes expression.

We tried to establish the respective effects of SRA and SRAP on the regulation of known endogenous target genes involved in breast cancer and ER signalling pathway. We transfected the four plasmid constructs into the T5 breast cancer cell line with lipofectamine (typical transfection reagent from invitrogen). However we confronted the same problem of low transfection efficiency. (see appendix 6 Figure1). Therefore, we plan to use lentiviral system to perform transient transduction by the different four SRA constructs mentioned above. After transfection, two micrograms of total RNA will be reverse transcribed and analyzed b real-time quantitative PCR using RT^2Profiler PCR Arrays (superArray biosciences corporation fredirick), which contains 84 optimized primer sets of different genes involved in breast cancer and ER signalling pathway. This technology provides the fastest way to scan the ER-target gene expression change after transfection of respective SRARNA and SRA protein.

Task3. To identify proteins interacting with SRAP and functions of SRAP interacted complex

a) To perform co-immunoprecipitation assays for identifying protein interacting with SRA proteins.

We immunoprecipitated SRAP-V5 from a previously described cell system consisting of MCF-7 cells over-expressing V5 tagged SRA protein (MCF-7 SRAP-V5 High.A). Whole cell extracts supplemented with V5 peptides, to compete out the immunoprecipitation of SRAP-V5, were used as negative control to identify proteins non-specifically precipitated. Tandem mass spectrometric analysis of immunoprecipitated samples identified a total of 110 unique proteins. We disregarded 23 of these proteins as non-specifically precipitated since they were present in both the immunoprecipitated sample and negative control. It is important to note that, SRAP was present among the remaining specifically precipitated 87 proteins. Using the functional gene classification software *(http://david.abcc.ncifcrf.gov/)*, we organized the proteins specifically associated with SRAP into distinct gene ontology functional categories, such as protein biosynthesis, protein degradation, proteins involved in transport and chaperones proteins. We assemble an additional group of proteins that might possibly be precipitated non-specifically with SRAP. There were 32 remaining proteins that could not be classified in any of the categories mentioned above. In order to build a hypothetical SRAP interactome, we submitted the 32 proteins to ingenuity pathways analysis software. Interestingly, 17 out of the 31 proteins (excluding SRAP) are nuclear proteins. Furthermore 11 out of the 18 nuclear proteins are involved in transcription and transcription regulation. (see appendix 3). The most striking finding of our study was the fact that the majority of the remaining proteins were nuclear and several of these have been shown to be involved in transcriptional regulation. Among these were MBD3 (Methyl-CPG binding domain protein 3, a member of the nucleosome remodeling and histone deacetylase complex (Nurd), BAF57 (a core subunit of the SWI/SNF chromatin remodeling complex) and YB-1 (Y-box binding protein, a transcription factor with multiple functions including co-repression and co-activation of other transcription factors). The high number of factors

involved in transcriptional regulation among SRAP interacting proteins strongly suggested that SRAP might also be involved in the modulation of transcription.

b) To validate the SRAP-protein interaction results obtained in task 3a.

By **GST pull down assay,** we have demonstrate recombinant GST-SRAP interact with the hp1α, mTIF1β, ERα, and ERβ in vitro(see appendix 6 Figure 2,3) All these are Transcription regulators
By **Panomics Array** Co-ip partners are nuclear and cytoplasmic. Nuclear partners are involved in transcription regulation. So we looked at the possible interaction of SRAP with transcriptional factors (Panomic) data. Recombinant SRAP is shown to interact with 40 different transcription factors, including ERβ and yy1(appendix 3 Table VII). Interestingly, we have also found that SRAP interacts with transcription factors with different binding affinities as assessed by the strength of the immuno-detected signals.

c) To validate the SRAP-interacting protein complex is associated with chromatin

These data above a),b) raised the possibility that SRAP is embedded in complexes regulating transcription events. Therefore we investigated whether SRAP could be associated to chromatin. SRAP V5 High.A cells were treated with formaldehyde and proteins cross-linked to chromatin were analyzed by Western blot using anti-SRAP, anti-Sp3. Both SRAP-V5 tagged and endogenous SRAP were found to be associated to chromatin. This association was not observed in the absence of cross-linking. As anticipated, both long (SP3L) and short (SP3M) SP3 isoforms (~ 100 kDa and ~60 kda respectively) known to be associated with chromatin, were also detected in the DNA bound protein fraction. The negative control used in this experiment is Gapdh (see appendix 3 Figure 5).

d) To validate what are functions of the SRAP-interacted complex

The observation that SRAP interacts with transcriptional regulators and transcription factors as well as associates with chromatin led us to hypothesize that it might itself participate in regulating transcription events. In order to establish the effect of SRAP recruitement on transcription sites, we investigated the consequence of SRAP fusion to the transcriptional activator GAL4-VP16. We showed that SRAP fusion to VP-16 decreased the activity of this strong transcriptional activator. In order to exclude any influence of SRA RNA, we generated an additional GAL4-VP16- SRA construct that contained a set of silent mutations (SDM1/7) demonstrated to nullify SRA RNA function without altering the SRAP coding sequence. Similarly to the wild type SRA, the SRA SDM1/7 also decreased VP-16 transcriptional activity suggesting that the transcriptional repressive activity of SRA was solely attributed to SRAP. Furthermore we determined that the transcriptional repressive activity of SRAP was sensitive to trichostatin A(HDAC inhibitor) treatment. These results thus suggest that SRAP possesses a transcriptional repressive activity possibly involving HDACs.

To determine if SRAP belongs to a complex containing HDAC activity, we immunoprecipitated SRAP-V5 from SRAP V5 High.A cells nuclear extracts. The SRAP-V5 immunoprecipitated sample contained significantly more HDAC activity than the control MCF-7 cells immunoprecipitated sample (see appendix 3 Figure 7).

We have recently reported intron-1 retention as a possible mechanism participating to the generation of coding and non coding SRA RNAs. In order to investigate the role of SRA intron-1 retention we decided to do the ***task4*** *using splice-switching-oligonucleotides targeted in junction of exon-1 and intron-1*(SRA-AS) *to increase the level of endogenous SRA intron-1 retention in the T5 breast tumor cell line. (details see appendix 4)*

Task4. To Tip the balance toward the production of more SRA intron-1 retention in breast cancer cell lines.

a) **To investigate change of endogenous SRA transcripts retaining intron-1 and fully spliced intron-1 fragment following treatment of T5 cells with SRA-AS**.

In T5 cells, we assessed the expression of intron-1 retaining RNA and fully spliced RNA of SRA by real-time PCR with a lower primer annealing in exon-3 and upper primers annealing with intron 1 or exon-1 after treatment of no oligonucleotide (Mock), SRA-AS or βgl-AS oligos (negative control). Four experiments were performed and the average modifications calculated). A significant ($p<0.05$, Student's t-test) average increase of 90% in the expression of SRA intron-1 retaining was observed in cells treated with SRA-AS 24h as compared to mock transfection. Inversely, a significant decrease of 70% in fully spliced SRA RNA expression was observed (appendix 4 Figure 7).

T5 cells were treated with increasing amounts (0.05, 0.1, 0.5 μM) of SRA-AS or βgl-AS oligos. Analysis of total cellular RNA by RT-TP-PCR reveals a dose-dependent increase in the proportion of full inton-1 retaining SRA transcripts in T5 cells treated with SRA-AS for 24h but not control βgl-AS. At 0.5uM SRA-AS (corresponding to 100% oligo uptake), an approximate 3-fold increase in the relative proportion of SRA-intron-1 retention was routinely achieved. (see appendix 4 figure 7)

To establish how long the increase in intron-1 retention upon treatment with SRA-AS lasted, time course experiments were performed as described in Materials and Methods. T5 cells were treated with no oligos (Mock) or with 0.5μM SRA-AS or βgl-AS oligos, and the relative expression of alternatively spliced SRA RNAs assessed by TP-PCR at t: 24 h, 48 h, and 72h. We found a significant increase (2.5 fold, $p<0.05$, Student's t-test) in relative intron-1 retention was observed at t: 24h upon SRA-AS treatment. A maximal 3.5-fold increase in the relative level of intron-1 retention was observed at t: 48 hours over that observed for time-matched mock transfected cells. This effect is maintained, albeit decreased at t: 72 hours(see appendix 4 Figure 6).

b) **To investigate how balance change between coding/non-coding endogenous SRA RNAs alters other gene expression in T5 breast cancer cell.**

Total RNA from T5 cells transfected with 0.5μM SRA-AS or control βgl-AS oligos was analyzed at t: 24h by real-time quantitative PCR using a Breast Cancer and Estrogen Receptor Signaling RT^2 Profiler™ PCR Array. Four independent experiments were performed as described in the Materials and Methods section. The expression of 56 genes was evaluated in cells treated with SRA-AS and βgl-AS oligos and differences in expression assessed using the Student's t-test. The expression of several genes was significantly modified A strong increase in the expressions of the urokinase plasminogen activator PLAU (ΔCt = 2.87, corresponding to a 729% expression compared to the control), gene intimately linked to invasion mechanisms as well as estrogen receptor beta (ΔCt = 1.73, corresponding to a 331% expression compared to the control) were observed. (see appendix 4 Figure 10).

Key research accomplishments:

1. We have determined trough protein arrays that SRAP is indeed able to directly interact with various transcription factors and transcription regulators.
2. We have established that SRAP is associated to chromatin in MCF-7 cells
3. We observed that SRA possesses a transcriptional repressive activity capable of inhibiting the GAL4-VP16 transcription activity. This SRAP transcriptional repressive potential is sensitive to trichostatin A treatment.
4. We determined that SRAP is able to co-immunoprecipitate HDAC activity.
5. 2'-O-methyl modified phosphorothioate oligoribonucleotides that target the junction of exon-1 and intron-1 serve as an efficient tool to increase endogenous SRA inton-1 retention in T5 cells. And increasing SRA intron-1 retention up-regulates the expression of several genes including PLAU and ERβ implicated in breast cancer progression and estrogenic signaling

In progress:
1. The results shown in task3 suggest that SRAP selectively associates with several transcriptional factors and is likely to have differential impact on the pathways involved. Therefore we are assessing SRAP action on each individual transcription factors and consequently SRAP role in the implicated pathways.

2. We are developing a lentiviral system carrying different SRA constructs, ERE-reporter constructs and ERs construct separately to characterize the respective action of SRA/SRAP on ER activity.

Reportable outcomes:

a) Publications:

Chooniedass-Kothari S, Hamedani MK, Troup S, Hube F, Leygue E (2005): The steroid receptor RNA activator protein is expressed in breast tumor tissues. Int.J.Cancer. (see appendix 1)

Hube F, Guo JM, Chooniedass-Kothari S, Cooper C, Hamedani MK, Leygue E. 2006: Alternative splicing of the first intron of the steroid receptor RNA activator (SRA) participates in the generation of coding and noncoding RNA isoforms in breast cancer cell lines. *DNA and Cell Biology 25*:418-428.(see appendix 2)

Chooniedass-Kothari S, Hamedani MK, Leygue E (2007): The Steroid receptor RNA activator protein (SRAP) belongs to a new family of transcriptional repressors. Submitted to JMB (see appendix 3)

Cooper C, Guo JM, Yan Y, Chooniedass-Kothari S, Leygue E(2007): Increasing Steroid Receptor RNA Activator (SRA) intron-1 retention in human breast cancer cells using modified oligonucleotides.To be Submitted to RNA (see appendix4)

b) poster presentation

Cooper C, Guo JM, Yan Y, Chooniedass-Kothari S ,Leygue E
Change in alternative splicing of the steroid receptor RNA activator (SRA) intron-1 modifies gene expression in T5 breast cancer.
AACR annual conference, Los angles, April 14-18.2007 (appendix5)

Conclusion

During this funding year of my project, we have determined trough protein arrays that SRAP is indeed able to directly interact with various transcription factors. Furthermore, we have established that SRAP is associated to chromatin in MCF-7 cells. we also examined the possible effect of SRAP recruitment on transcription using the potent GAL4-VP16 hybrid transcription activation system. We observed that SRA possesses a transcriptional repressive activity capable of inhibiting the GAL4-VP16 transcription activity. This SRAP transcriptional repressive potential is sensitive to trichostatin A (a HDAC inhibitor) treatment. And SRAP is able to co-immunoprecipitate HDAC activity. Meanwhile, we have also investigate the possible mechanism of intron-1 retention as a participating to the generation of coding and non coding SRA RNAs. We found 2'-O-methyl modified phosphorothioate oligoribonucleotides that target the junction of exon-1 and intron-1 serve as an efficient tool to increase endogenous SRA inton-1 retention in T5 cells. And increasing SRA intron-1 retention up-regulates the expression of several genes including PLAU and ERβ implicated in breast cancer progression and estrogenic signaling

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6. Hube F, Guo JM, Chooniedass-Kothari S, Cooper C, Hamedani MK, Leygue E. 2006: Alternative splicing of the first intron of the steroid receptor RNA activator (SRA) participates in the generation of coding and noncoding RNA isoforms in breast cancer cell lines. *DNA and Cell Biology 25*:418-428.

7. Chooniedass-Kothari S, Hamedani MK, Leygue E (2007): The Steroid receptor RNA activator protein (SRAP) belongs to a new family of transcriptional repressors. Submitted to JMB

8. Cooper C, Guo JM, Yan Y, Chooniedass-Kothari S ,Leygue E(2007): Increasing Steroid Receptor RNA Activator (SRA) intron-1 retention in human breast cancer cells using modified oligonucleotides.To be Submitted to RNA

Int. J. Cancer: **118,** 1054–1059 (2006)

SHORT REPORT

The steroid receptor RNA activator protein is expressed in breast tumor tissues

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The steroid receptor RNA activator (SRA) was originally described as the first functional non-coding RNA able to specifically coactivate the activity of steroid receptors. We previously demonstrated the existence in breast cancer cell lines of new SRA isoforms that, as opposed to the first cloned SRA RNA, encode for a 236–amino acid protein, SRAP. To investigate the possible implications of the coding SRA RNA and SRAP expression on breast cancer progression, we examined by Western blot analysis 74 primary breast tumors of patients subsequently treated with tamoxifen. Patients whose primary tumors were positive for SRAP expression ($n = 24$) had a significantly (Kaplan-Meier survival curve $p = 0.047$) lower likelihood of dying from recurrent disease than SRAP-negative patients ($n = 50$). We generated 2 cell lines, SRAP-V5-High.A and SRAP-V5-High.B, by stably overexpressing SRAP in the estrogen receptor-positive MCF-7 breast cancer cell line. Transient transfection experiments, performed using a luciferase reporter gene under the control of an estrogen-responsive element, revealed decreased sensitivity to estradiol but no additional sensitivity to tamoxifen in SRAP-overexpressing cells. Overall, our data suggest that the presence of both coding SRA RNA and its corresponding SRAP modifies the activity of estrogen receptor in breast cancer cells and that SRAP could be a new clinical marker for breast cancer. Further studies are needed to define the respective mechanisms of action and the roles of SRA RNA and protein in breast tumorigenesis and tumor progression.

Key words: steroid receptor coactivator; human breast tumor; steroid receptor coactivator protein

Through its action on breast epithelial cells, estrogen not only controls the growth and the development of normal mammary gland but also promotes breast tumorigenesis and breast cancer progression.[1] The biological action of estrogen is mainly mediated through two ERs, α and β, which act as ligand-dependent transcription factors.[2,3] While estrogen initially plays a pivotal role in the activation of ERs, the transcriptional activation of target genes is ultimately determined by interactions between receptors and regulatory molecules known as coactivators and corepressors, which respectively stimulate or inhibit ER activity.[4] SRA differs from all previously characterized coactivators as it was originally identified as a functional non-coding RNA molecule.[5] SRA mechanisms of action have since become the focus of extensive investigation. SRA was shown to contain a core RNA sequence necessary and sufficient to mediate steroid receptor activity[6] through interactions with several proteins including the coactivator/corepressor SHARP,[7] SRC1,[5] and the AF-1-specific activator p72/p68 protein.[8] Post-transcriptional modifications of SRA have also been shown to participate in the ability of this RNA to modulate receptor activity.[9] We established that SRA RNA was differentially expressed in normal and in breast tumor tissue and suggested that SRA RNA could be involved in mechanisms underlying breast tumorigenesis and breast tumor progression.[10] The observation by Lanz *et al.*[11] of multiple proliferation anomalies in the overexpressing noncoding SRA RNA mammary glands of transgenic mice corroborates this hypothesis.

While all these studies refer to SRA as a noncoding RNA, we have previously demonstrated the existence of coding SRA RNA isoforms and corresponding endogenous SRA proteins,[12] highly conserved in vertebrates and expressed in breast cancer cell lines.[13,14] To date, no data are available on the possible role of the coding SRA RNA or SRAP in breast cancer cells or on their expression in human breast tumor tissues. Here, we investigated SRAP expression in a cohort of ER-positive primary breast tumors from patients subsequently treated with tamoxifen and examined the effect of SRAP overexpression on ERα activity in MCF-7 mammary tumor cells.

Material and methods

Human breast tissues and cell lines

Seventy-four primary breast tumors were selected from the Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). Tumors corresponded to cases associated with node-negative status that were treated by surgery with or without radiation therapy and then tamoxifen endocrine therapy. All tumors were ER-positive (ER levels ranging 4–247 fmol/mg protein, median 45.5) and spanned a wide range of PR levels (2.4–444 fmol/mg protein, median 31). Notthingham grade was known for 66 tumors, which were assigned to low ($n = 23$, scores 3–5), moderate ($n = 35$, scores 6–7) or high ($n = 8$, scores 8–9) categories. MCF-7 cells were stably transfected with the pCDNA.3.1-V5-His expression vector alone (control cell line) or containing a coding SRA cDNA to generate the SRAP-V5-High.A, SRAP-V5-High.B and SRAP-V5 low cell lines, as previously reported.[14]

Western blot analysis

Total proteins were extracted from cells[14] or breast tumor tissues[15] and analyzed by Western blot as previously described.[13–15] Four primary antibodies, a rabbit polyclonal anti-SRAP antibody,[14] a mouse anti-ER MAb (NCL-ER 6F11/2; Novocastra, Newcastle, UK), a mouse anti-PR MAb (NCL-PGR 312, Novocastra) and a mouse anti-β-actin MAb (A5441; Sigma, Oakville, ON), were used at dilutions of 1:1,000, 1:1,000, 1:1,000 and 1:5,000, respectively. Preincubation of the primary anti-SRAP with its corresponding peptide was performed as described previously.[14]

To examine PR expression, MCF-7 cont, SRAP-V5-High.A, SRAP-V5-High.B and SRAP-V5-Low were grown for 6 days in serum-free phenol red-free DMEM supplemented with apotransferrin and BSA. Cells were then treated with either ethanol (vehicle) or estradiol (10^{-8} M) for 4, 24 and 48 hr. Cells were then lysed, and identical amounts of total protein extracts were analyzed as described above. To ensure equal loading, gels were stained with Coomassie blue.

Abbreviations: ER, estrogen receptor; ERE, estrogen-responsive element; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAb, monoclonal antibody; PR, progesterone receptor; SHARP, SMRT/HDAC-associated repressor protein; SRA, steroid receptor RNA activator; SRAP, protein encoded by the steroid receptor RNA activator; SRC1, steroid receptor coactivator-1; TFF1, trefoil factor 1.

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RT-PCR analysis

MCF-7 cont, SRAP-V5-High.A, SRAP-V5-High.B and SRAP-V5-Low cells were grown for 6 days in serum-free phenol red-free DMEM supplemented with apotransferrin and BSA and subsequently treated with either ethanol (vehicle) or estradiol (10^{-8} M) for 15 and 60 min. Total RNA was isolated from these cells using the Eppendorf (Hamburg, Germany) RNA isolation kit. cDNA was synthesized using MMLV reverse transcriptase (Invitrogen, Burlington, ON) as described in the manufacturer's instructions. cDNAs were then amplified with platinum Taq polymerase (Invitrogen) as previously described.[16] Primers to TFF1 were upper 5′-CTGGGGCACCTTGCATTTTCC-3′ and lower 5′-CGGGGG-GCCACTGTACACGTC-3′,[17] and those to GAPDH were upper 5′-ACCCACTCCTCCACCTGG-3′ and lower 5′-CTCTTGTGC-TCTTGCTGGG-3′.

PCR products were separated electrophoretically on 2% agarose gels and subsequently stained with ethidium bromide. Gels were visualized under UV light on a GelDoc2000/ChemiDoc System (Bio-Rad, Richmond, CA).

Cell transfection experiments

Transfection experiments were performed as previously described[18] with small modifications. Briefly, cells grown in phenol red-free DMEM supplemented with 5% charcoal-stripped FBS for 48 hr were transfected for 4 hr with 1 μg of ERE-luciferase plasmid (containing a vitellogenin ERE, GGTCACTGTGACC site upstream of the firefly luciferase cDNA) and 0.1 μg of renilla luciferase reporter vector (Promega, Madison, WI) using the lipofectamine reagent (Invitrogen). Cells were then treated with either ethanol (vehicle), estradiol (10^{-6}–10^{-10} M) and/or 4-hydroxytamoxifen (Sigma, 10^{-6}–10^{-9} M) for 24 hr. Cells were lysed in 200 μl of cell culture lysis buffer (Promega), and lysates were analyzed for luciferase and renilla luciferase activities according to the manufacturer's protocol (Promega).

Statistical analysis

Transfection results were normalized by dividing ERE luciferase activities by their corresponding renilla luciferase activities. For each treatment, the relative luciferase activity was used to calculate fold induction (ratio of value for a treatment and the corresponding value for ethanol treatment). Results are representative of at least 3 independent experiments. Significant differences were assessed using Student's *t*-test. Error bars represent SEM.

Tumor cases were classified as SRAP-positive or SRAP-negative following independent assessment of the corresponding Western blot signal by 2 investigators. Statistical differences in ER/PR or Nottingham grade between the 2 groups were tested using the Mann-Whitney test (2-tailed) or the χ^2 test, respectively. Relapse-free survival was defined as the time from initial surgery to the date of clinically documented local or distant disease recurrence or death attributed to breast cancer. Overall survival was defined as the time from initial surgery to the date of death attributed to breast cancer. Deaths caused by other known or unknown causes were censored. The association between SRAP expression and relapse or survival was assessed by the Kaplan-Meier method.

Results

Differential expression of SRAP in breast tumor tissues

To investigate the possible relationship between SRAP expression and known prognostic markers, we performed Western blot analysis on a series of proteins extracted from 74 different ER-positive breast tumors. A strong background signal (50 kDa), still present with the neutralizing peptide, was observed in all tumors (Fig. 1*a,b*).

SRAP signal, which disappeared when the antibody was preincubated with the corresponding peptide, was observed in some (24 tumors, *e.g.,* lanes 1, 2, 8), but not all (50 tumors, *e.g.,* lanes 3, 4, 5, 7), tumors (Fig. 1*a,b*). As expected, the size of the bands varied around 30 kDa, likely as a result of the genetic background (homozygous/heterozygous for the different alleles) of the patients.[14] A band of approximately 25 kDa, not previously seen in breast cancer cell lines,[14] was also specifically recognized in 34 cases (*e.g.,* lanes 1, 2, 4–8 of Fig. 1*a*).

Neither ER nor PR levels were significantly different (Mann-Whitney rank sum test $p > 0.05$) between SRAP-positive ($n = 24$, median ER 74.5, PR 30.5 fmol/mg protein) and SRAP-negative ($n = 50$, median ER 39, PR 32.5 fmol/mg protein) subgroups. Similarly, no significant relationship (χ^2 test $p > 0.05$) was found between SRAP expression and histologic tumor grade distribution (low grade $n = 7$, $n = 16$; moderate grade $n = 9$, $n = 26$; high grade $n = 5$, $n = 3$ for SRAP-positive and -negative subgroups, respectively).

When SRAP expression was considered in relation to recurrence, no significant difference was seen ($n = 9$ events for SRAP negative patients and 3 events for SRAP-positive patients, Fig. 1*c*). In contrast, when considered in relation to outcome (Fig. 1*d*), a significant (Kaplan-Meier $p = 0.044$) association was found with undetectable level of SRAP expression and poor survival ($n = 7$ events for SRAP-negative and 0 events for SRAP-positive).

No correlation was found between expression of the 25 kDa band and any tumor or patient characteristics (data not shown).

Clones stably expressing SRAP-V5 recombinant protein

We stably transfected MCF-7 mammary cancer cells, known to express high levels of endogenous ER, with a construction consisting of coding SRA RNA able to encode a fusion protein, SRAP-V5-tag.[14] Several clones were selected and protein extracts analyzed by Western blot using an anti-SRA antibody previously shown to recognize both endogenous SRAP (approx. 30 kDa) and V5-tagged SRAP (approx. 35 kDa).[14] As shown on Figure 2*a,* clones expressing detectable levels (SRAP-V5-High.A and SRAP-V5-High.B) or not detectable levels (SRAP-V5-Low) of the recombinant SRAP-V5 protein were obtained. All cell lines, including control MCF-7 stably transfected with vector alone, expressed identical levels of endogenous SRAP (Fig. 2*a*), ER (Fig. 2*b*) and PR isoforms A (112 kDa) and B (83 kDa) (Fig. 2*c*), as assessed by Western blot.

Decreased ligand-dependent transcriptional activity of ER in SRAP-V5-overexpressing cells

To establish whether the ER signaling pathway was altered in cells overexpressing SRAP, an ERE-luciferase reporter vector was transiently transfected in all 4 cell lines described above. Cells were then treated with ethanol (vehicle), estradiol (10^{-8} M) or 4-hydroxytamoxifen (10^{-6} M) for 24 hr and luciferase activity was measured, as described in Material and methods. Luciferase activities of the 4 cell lines were identical when cells were treated with ethanol or 4-hydroxytamoxifen (data not shown and Fig. 3*a*). However, upon estradiol (10^{-8} M) treatment, cells overexpressing SRAP-V5 (SRAP-V5-High.A and SRAP-V5-High.B) showed significantly lower induction (approx. 19-fold, Student's *t*-test $p < 0.03$) of ERα transcriptional activity compared to control cells (approx. 39-fold, Fig. 3*a*). In contrast, even though the reporter gene induction was slightly lower (approx. 32-fold) in SRAP-V5-Low cells compared to control cells (approx. 39-fold), this difference did not reach statistical significance ($p > 0.05$).

To establish whether the decreased activation of ER in SRAP-V5-overexpressing cells was dependent on the dose of estradiol used, we transiently transfected all 4 cell lines with an ERE reporter gene and treated them with increasing amounts of estradiol (10^{-6}–10^{-10} M). Lower activation of the reporter gene in SRAP-V5-overexpressing cells compared to control cells (Fig. 3*b*) was observed for all concentrations of estradiol used. Differences between the SRAP-overexpressing and control cell lines were statistically significant ($p < 0.02$) at 10^{-8}, 10^{-9} and 10^{-10} M. We suspect treatment with higher concentrations (10^{-6} and 10^{-7} M) had toxic effects on cells that could have led to lower reproducibility between experiments.

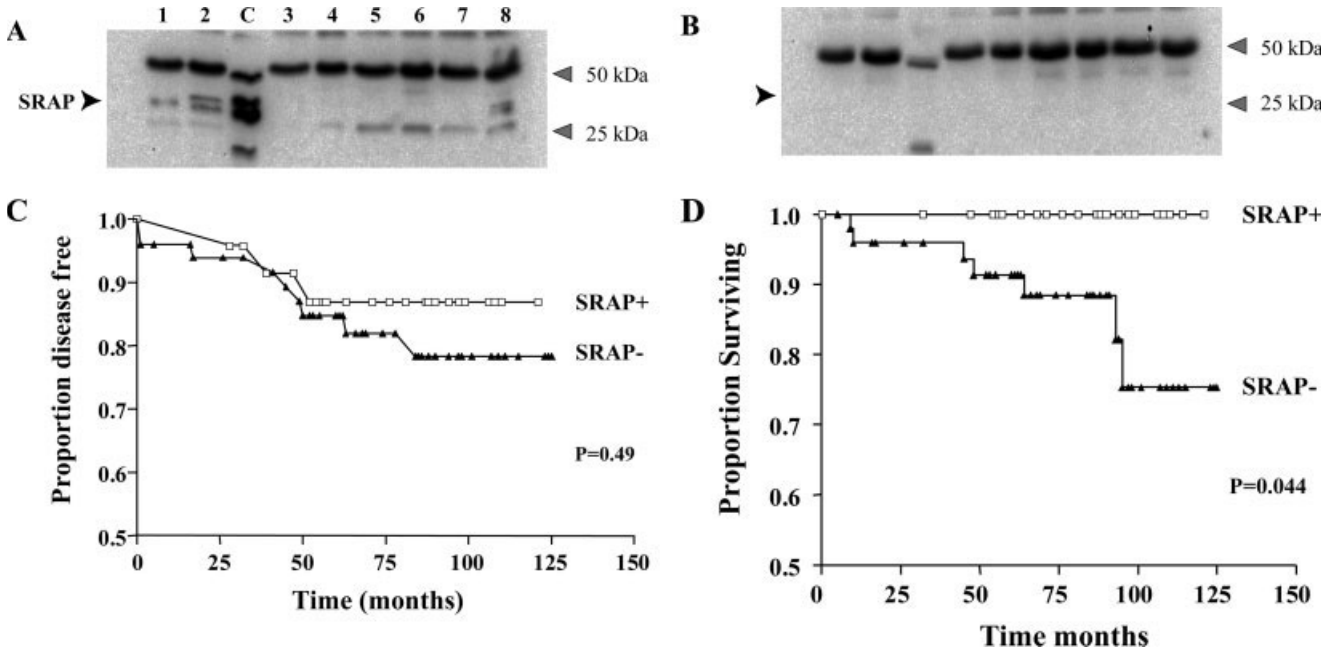

FIGURE 1 – SRAP expression correlates with overall better survival in ER-positive breast cancer patients. Proteins were extracted from a cohort of 74 ER-positive tumors and analyzed by Western blot for SRAP expression as described in Material and methods. *(a)* Representative panel showing Western blot for tumors 1–8. C, SRAP-V5-High.A cells. *(b)* Western blot performed in parallel and incubated with an anti-SRA antibody premixed with the neutralizing peptide. *(c,d)* Kaplan-Meier graphs for time to progression and overall survival, respectively, with regard to SRAP expression.

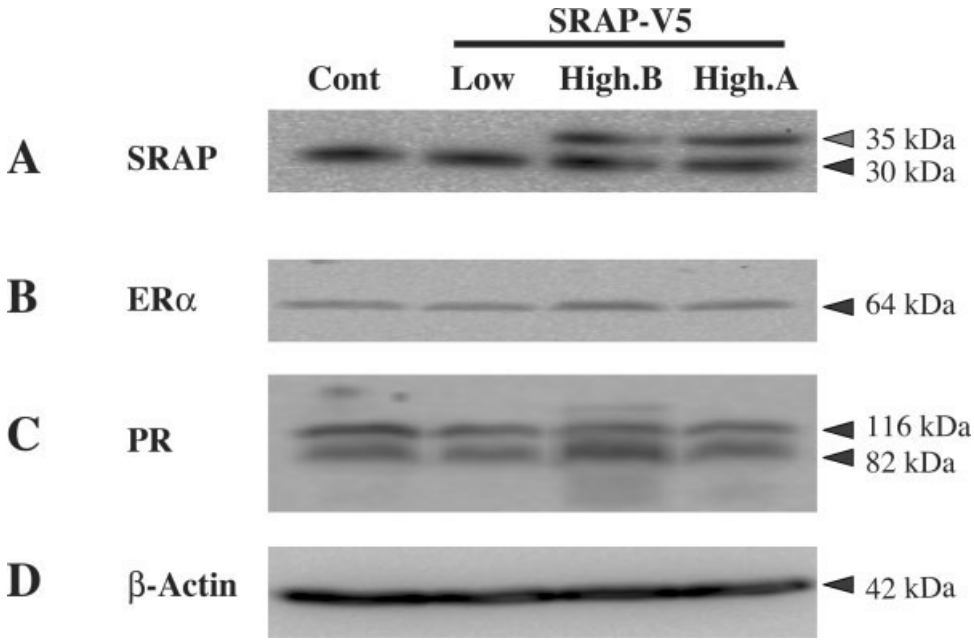

FIGURE 2 – Western blot analysis of MCF-7 cells stably transfected with SRAP-V5 cDNA. MCF-7 breast cancer cells were stably transfected with SRAP-V5 cDNA (SRAP-V5) or empty vector, and total protein extracts were analyzed by Western blot as described in Material and methods. Antibodies consisted of anti-SRAP *(a)*, anti-ERα *(b)*, anti-PR *(c)* and anti-actin *(d)*. Two high (High.A and -.B) and one not detectable (Low.A) SRAP-V5 expressors were selected for further analysis.

Similar tamoxifen sensitivity in SRAP-V5-overexpressing and control cell lines

To determine whether SRAP overexpression potentiates the inhibitory effect of 4-hydroxytamoxifen, SRAP-V5-High.A, SRAP-V5-High.B, SRAP-V5-Low and control cells transiently transfected with an ERE-luciferase reporter vector were treated with 10^{-8} M estradiol supplemented with increasing amounts of 4-hydroxytamoxifen (10^{-9}–10^{-6} M). Reporter gene activities were lower in SRAP-overexpressing cells compared to activities observed for the corresponding treatment in control cells (Fig. 3*c*). However, in all cell lines, the first efficient concentration of 4-hydroxytamoxifen able to significantly ($p < 0.05$) decrease estradiol-dependent induction was 10^{-7} M (Fig. 3*c*).

Higher PR expression upon estradiol stimulation in SRAP-V5-overexpressing cells

It was important to determine whether the difference in the response to estradiol observed between high and low SRAP-expressing MCF-7 cells seen with the luciferase reporter assay could also be observed during the induction of known ER target

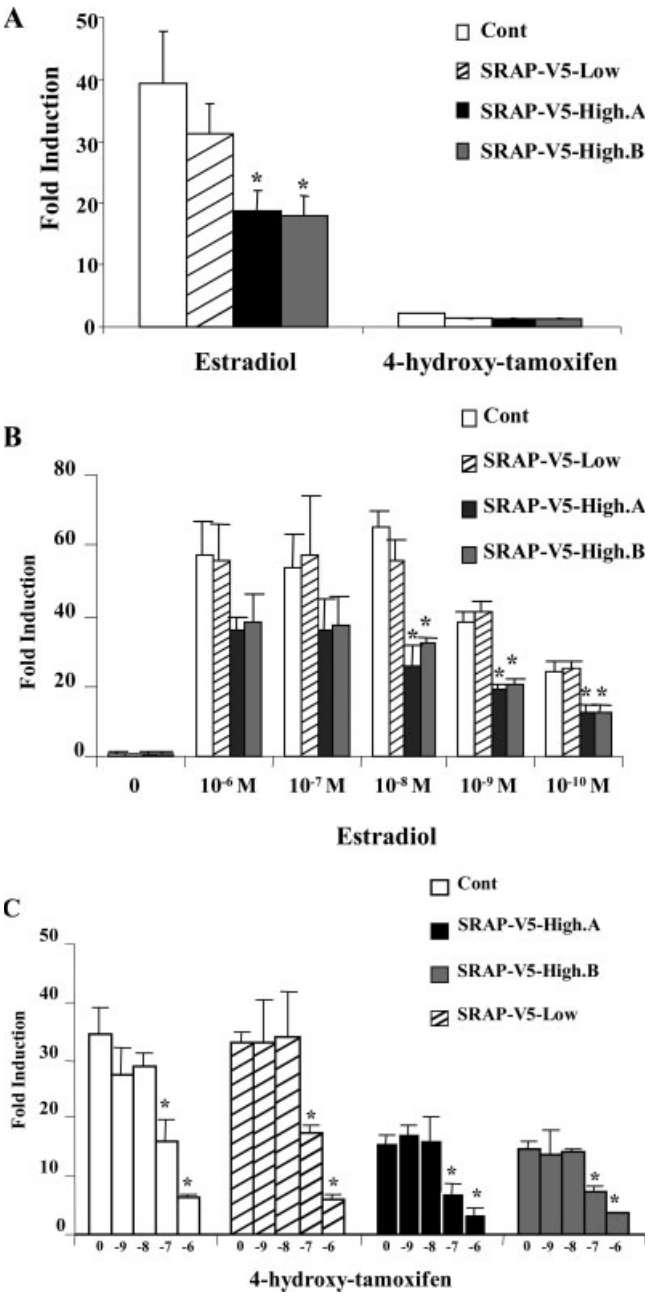

FIGURE 3 – Decreased activation of an ERE-luciferase reporter gene in SRAP-V5-overexpressing cells. MCF-7 cells expressing detectable (SRAP-V5-High.A and -.B) or not detectable (SRAP-V5-Low and Cont) levels of SRAP-V5 recombinant protein were transiently transfected with an ERE-luciferase reporter gene and subsequently treated with *(a)* ethanol, 10^{-8} M estradiol or 10^{-6} M 4-hydroxytamoxifen; *(b)* 10^{-6}–10^{-10} M estradiol; or *(c)* 10^{-8} M estradiol + 0, 10^{-9}, 10^{-8}, 10^{-7} or 10^{-6} M 4-hydoxytamoxifen for 24 hr. Results correspond to the average fold induction (ratio between luciferase values during ligand treatment and corresponding ethanol treatment) of at least 3 independent experiments. Bars = SEM. Cont, MCF-7 cells stably transfected with vector alone. **(a,b)* Statistically significant difference ($p < 0.05$, Student's *t*-test) between the fold induction obtained for SRAP-V5-overexpressing clones and the corresponding fold induction for control cells. **(c)* Fold inductions for specific tamoxifen concentrations that were statistically lower ($p < 0.05$, Student's *t*-test) than the corresponding fold induction at 10^{-8} M estradiol for each cell line.

genes. To address this question, SRAP-V5-High.A, SRAP-V5-High.B, SRAP-V5-Low and control cells were grown in serum-free media and subsequently treated with 10^{-8} M estradiol for 4, 24 and 48 hr. Proteins were extracted and identical amounts of total protein lysates were analyzed by Western blot for PR expression, as described in Material and methods. As shown in Figure 4*a,* PR protein was extremely low or even undetectable in all 4 cell lines when grown in serum-free medium for 6 days (t = 0) and when treated with vehicle alone. Interestingly, upon 24 and 48 hr of estradiol stimulation, SRAP-V5-High.A and -High.B expressed noticeably higher PR levels than the control cell line.[19] To ensure equal loading, SDS-PAGE gels were stained with Coomassie blue, and the intensity of the staining was shown to be identical in all cell lines (data not shown). As seen in Figure 4*a,* the SRAP-V5-Low cell line also had a higher PR induction at 24 and 48 hr than the control cell line. However, PR expression was considerably lower in the SRAP-V5-Low cell line compared to the SRAP-V5-High.A and -High.B cell lines at 48 hr.

Similar estradiol-dependent TFF1 mRNA induction in SRAP-V5-overexpressing and control cells

TFF1 is another well-characterized ER target gene, expression of which was increased by estradiol as early as 1 hr after treatment of MCF-7 cells.[20] To determine whether the estradiol-dependent induction of TFF1 is differentially regulated in SRAP-overexpressing cells, SRAP-V5-High.A, SRAP-V5-High.B, SRAP-V5-Low and control cells were grown in serum-free media and subsequently treated with 10^{-8} M estradiol for 15 and 60 min. Total RNA was extracted, reverse-transcribed and analyzed by RT-PCR using primers recognizing TFF1 cDNA as described in Material and methods. As shown in Figure 4*b,* a similar increase in TFF1 mRNA levels was observed in all 4 cell lines upon 60 min of 10^{-8} M estradiol treatment.

Discussion

To date, all functional studies on SRA have focused only on its RNA aspect and were performed in transient expression systems. Here, we establish the existence of the corresponding SRAP in breast tumor tissues and examine the possible implication of SRAP expression on the ER signaling pathway.

SRAP was detected by Western blot analysis in 24 of 74 (32%) cases, migrating at around 30 kDa and appearing as either a single band or a doublet. We suspect that the diverse band pattern observed in breast tumor tissues results from the different genetic background of the patients. Indeed, we have previously demonstrated the existence of 3 SRAP isoforms, with SRA isoform 3 migrating slightly slower than the other two.[14] The differences in SRAP migration in the tumor samples could therefore be due to homozygosity/heterozygosity for the different SRA isoforms and/or the differential use of either the first or second methionine as described previously.[13]

In addition to the expected 30 kDa band, we observed in 34 of 74 cases (45%) a 25 kDa band specifically recognized by our antibody. Although the theoretical size of SRAP is 25 kDa, we had previously never detected a 25 kDa SRAP in any of the breast cancer cell lines grown *in vitro* and analyzed by Western blot.[14] This new form of SRAP seen in breast tumor tissues cannot be attributed to an alternative translation starting at the second methionine as a 12 amino acid difference would not account for a shift in migration by 5 kDa. Similarly, the 25 kDa form cannot correspond to an alternative translation starting at the third methionine (at amino acid position 75) since this form of SRAP would not be detected by our antibody targeted against amino acids 20–34. We suspect this 25 kDa band has distinct posttranslational modifications from the ones observed on the 30 kDa SRAP. Whether both are expressed by the same cells remains to be determined. Antibodies directed against different regions of SRAP will be generated to address these issues.

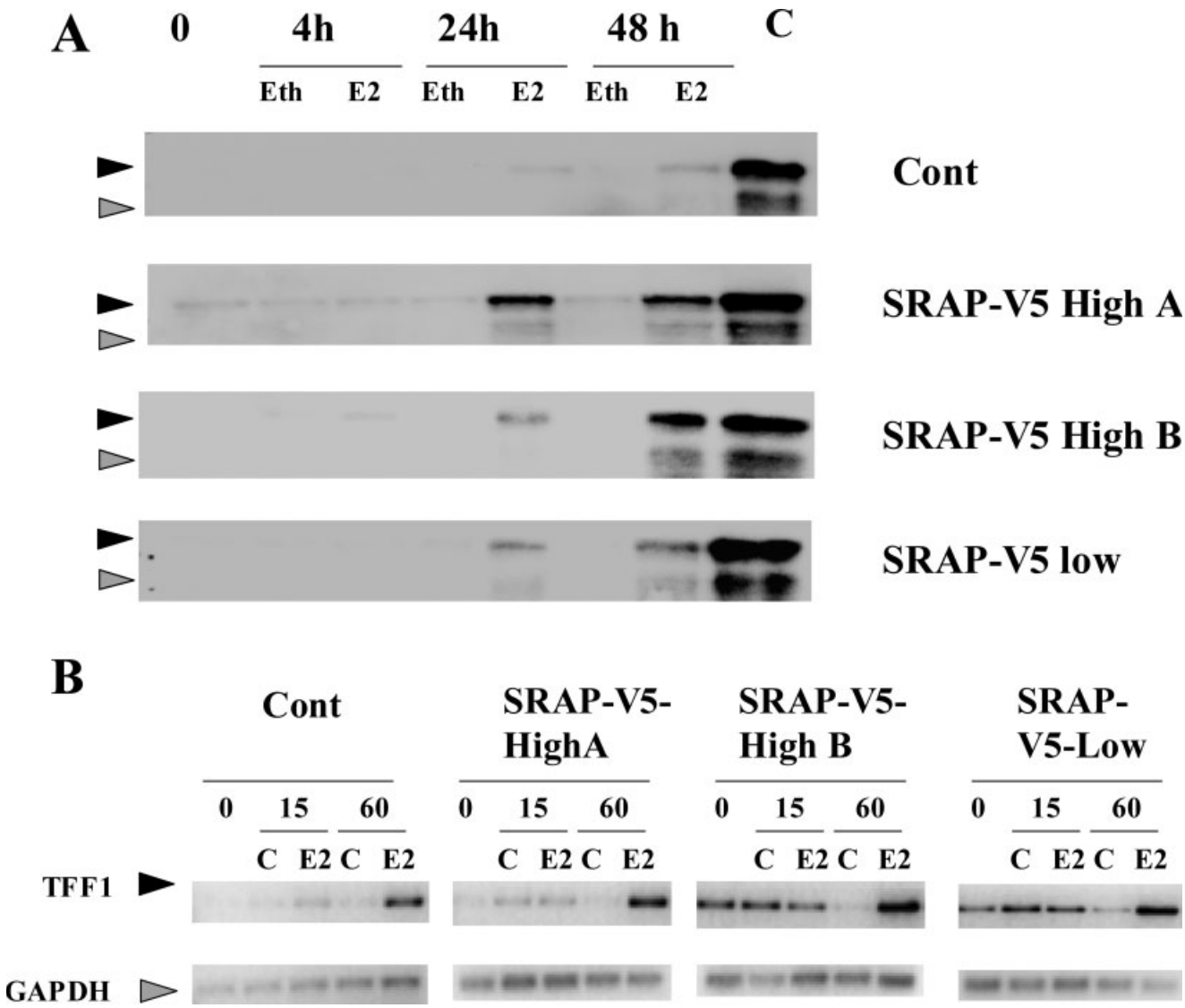

FIGURE 4 – SRAP-V5-overexpressing cells have higher PR, but not TFF1, expression upon estradiol stimulation. MCF-7 cells expressing detectable (SRAP-V5-High.A and -.B) or not detectable (SRAP-V5-Low and Cont) levels of SRAP-V5 recombinant protein were grown in serum-free medium for 6 days and subsequently treated with ethanol or 10^{-8} M estradiol for *(a)* 4, 24 and 48 hr or *(b)* 15 or 60 min as described in Material and methods. *(a)* Cells were lysed, and identical amounts of total protein extract were analyzed by Western blot for PR expression. An identical amount (100 μg) of a lysate positive for PR expression was used as positive control (C). Black arrows indicate PR isoform A (112 kDa); gray arrows indicate PR isoform B (82 kDa). *(b)* Cells were lysed, and extracted RNAs were reversed-transcribed and then analyzed for TFF1 and GAPDH expression by PCR as indicated in Material and methods.

No significant correlations were found between the detection of any SRAP (30 kDa or 25 kDa) and levels of ER or PR or tumor grade. Similarly, no correlation was observed between the reoccurrence of disease and SRAP expression. We have, however, found that expression of the 30 kDa SRAP correlated with overall better survival in ER-positive patients subsequently treated with tamoxifen. This suggests that SRAP could be a new independent prognostic marker that might predict disease outcome. In other words, detection of SRAP in the primary tumor could be a marker of a ''less aggressive'' form of cancer. Further analyses, performed on larger cohorts of patients associated with different tumor subgroups, are needed to corroborate this hypothesis.

The observed correlation between SRAP expression and overall better survival in ER-positive patients prompts us to examine further the impact of the coding SRA RNA and consequently SRAP on the ER signaling pathway. Here, we show that overexpression of SRAP in breast cancer MCF-7 cells results in decreased responsiveness to estrogen (for all concentrations used), as assessed by activation of a transiently expressed ERE-luciferase reporter gene. This decrease cannot be attributed to a change in ER-α expression since similar levels of this receptor are detected in control and SRAP-overexpressing cells.

SRA RNA overexpression had previously been shown to potentiate ER and PR transcriptional activities.[5,18] We therefore did not expect to observe decreased ER activity in cells stably overexpressing SRA RNA. To our knowledge, all the SRA sequences used by others when investigating SRA RNA function lacked the first 2 starting methionines and were consequently unable to encode SRAP.[5–7,11] The SRA sequence used here contains 32 additional 5′ end base pairs with 2 putative starting methionines and therefore has the capacity to initiate the translation of either a 236– or a 224–amino acid SRAP.[14] This coding RNA is expected to function as an ER-activating RNA since it contains an intact SRA core sequence previously shown to be necessary and suffi-

cient for SRA RNA to function as a non-coding RNA.[5] Our luciferase reporter assays therefore indicate that the concurrent coding SRA RNA and protein expressions result in a significant reduction of ER activity. We observed such opposite action of the coding SRA RNA from the previously reported steroid receptor coactivating function of the noncoding SRA RNA. We therefore suspect that SRAP expression is responsible for this apparent lower ER activity. Further studies are, however, needed to demonstrate this hypothesis. In addition, our results suggest that the concurrent expression of coding SRA RNA/SRAP selectively modifies the activity of estradiol-stimulated ER but does not affect the ER sensitivity to tamoxifen. This fits the observation that patients whose primary tumors expressed SRAP did not have a lower incidence in reoccurrence of the disease. Indeed, in light of our reporter gene assay, tumor cells expressing high levels of SRAP are not suspected to respond better to tamoxifen.

Similar PR levels were observed in control and SRAP-overexpressing cells when grown in complete medium. PR is an ER target gene, and an apparent decrease in ER activity, through overexpression of SRAP, was expected to lead to lower PR levels in these cells. Surprisingly however, when cells were grown in serum-free medium and subsequently treated with estradiol, PR expression was induced faster in the SRAP-V5-overexpressing cells. Although noncoding SRA RNA has previously been shown to increase PR expression and activity,[5] it is premature to attribute the increased PR levels to expression of either the RNA or protein. Indeed, our MCF-7 cell model reflects a more complex system with concurrent actions of both SRA RNA and protein. This model, although more comprehensive, does not allow separation and analyses of the SRA RNA and protein functions. In addition, it is now increasingly apparent that coregulating molecules altering ER activity do not have a global effect but rather distinct outcomes on individual target genes.[21] In support of this concept, we observed that the estradiol-mediated regulation of the 2 ER target genes studied (*PR* and *TFF1*) was dissimilar in the SRAP-V5-overexpressing and control cells. Indeed, while the estradiol-mediated induction of PR was evidently distinct between the SRAP-overexpressing and control cell lines, no difference in TFF1 induction was observed between the cells. Furthermore, although noncoding SRA RNA has been shown to act as an ER activator, a recent study has shown that it is only able to activate distinct ER target gene promoters.[21] Additional studies are needed and will be performed to dissect separately the exact mechanisms of action of the SRAP and SRA RNA and subsequently analyze their respective actions on individual ER target genes. Nonetheless, our reporter assays and analyses of PR expression have demonstrated that expression of the coding SRA RNA leads to alteration in the ER signaling pathway distinct from the previously reported effect of the noncoding SRA RNA.

To date, all functional studies on SRA have focused only on its RNA aspect. Here, we establish the existence of the corresponding SRAP in breast tumor tissues and examined the possible implication of a coding SRA RNA and consequently SRAP expression on the ER signaling pathway and breast cancer progression. The discovery that SRAP might itself also be implicated in the ER signaling pathway and that its expression correlates with disease outcome emphasizes the need to actively probe the exact mechanisms of action of this increasingly complex but promising bifaceted molecule. Indeed, additional studies, examining the separate and concurrent functions and regulations of SRA RNA and SRAP, are essential to establish the clinical potential of these bifaceted molecules in the treatment of breast cancer.

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APPENDIX2: DNA AND CELL BIOLOGY
Volume 25, Number 7, 2006

Pp. 418-428

Alternative Splicing of the First Intron of the Steroid Receptor RNA Activator (SRA) Participates in the Generation of Coding and Noncoding RNA Isoforms in Breast Cancer Cell Lines

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ABSTRACT

The Steroid Receptor RNA Activator 1 (SRA1) has originally been described as a noncoding RNA specifically activating steroid receptor transcriptional activity. We have, however, identified, in human breast tissue, exon-1 extended SRA1 isoforms containing two initiating AUG codons and encoding a protein we called SRAP. We recently reported a decreased estrogen receptor activity in breast cancer cells overexpressing SRAP, suggesting antagonist roles played by SRA1 RNA and SRAP. SRA1 appears to be the first example of a molecule active both at the RNA and at the protein level. No data are currently available regarding the mechanisms possibly involved in the generation of coding and noncoding functional SRA1 RNAs. Using 5′-Rapid Amplification of cDNA Extremities (5′-RACE), we have herein identified several putative transcription initiation sites surrounding the second methionine codon and used to generate coding SRA1 transcripts. In the process, we also identified an alternatively spliced noncoding SRA1 transcript still containing an intron-1 sequence. Using targeted RT-PCR approaches, we confirmed the presence in breast cancer cell lines of SRA1 RNAs containing a full as well as a partial intron-1 sequence and established that the relative proportion of these RNAs varied within breast cancer cell lines. Using a "minigene" strategy, we also showed that artificial RNAs containing the SRA1 intron-1 sequence are alternatively spliced in breast cancer cell lines. Interestingly, the splicing pattern of the minigene products parallels the one of the endogenous SRA1 transcripts. Altogether, our data suggest that the primary genomic sequence in and around intron-1 is sufficient to lead to a differential splicing of this intron. We propose that alternative splicing of intron-1 is one mechanism used by breast cancer cells to regulate the balance between coding and functional noncoding SRA1 RNAs.

INTRODUCTION

THROUGH ITS MITOGENIC ACTION on breast epithelial cells, estrogen not only controls the growth and the development of the normal mammary gland, but also promotes breast tumorigenesis and breast cancer progression (for a review, see Jensen and Jordan, 2003). This estrogenic action is mainly mediated through two estrogen receptors (ERs) alpha and beta (Green *et al*., 1986; Mosselman *et al*., 1996), that belong to the steroid/thyroid/retinoic acid receptors superfamily and act as ligand-dependent transcription factors (Evans, 1988). Over the last few years, it has become apparent that the balance between coactivators and corepressors ultimately controls steroid receptor action in a given tissue (Shibata *et al*., 1997; Jordan and Morrow, 1999). This balance is modified during breast tumorigenesis, and a search for possible means to readjust it has therefore started worldwide with the hope to develop novel therapeutic strategies (McKenna *et al*., 1999a, 1999b; McKenna and O'Malley, 2001; Gao *et al*., 2002; Gao and Nawaz, 2002).

In 1999, Lanz *et al*. (1999) identified three human transcripts

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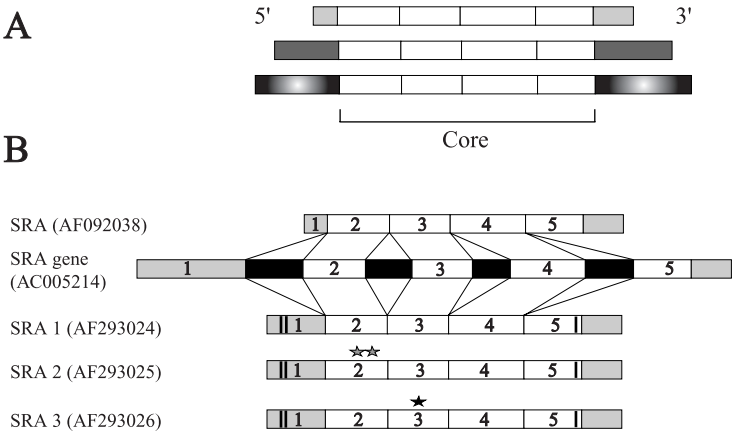

FIG. 1. Schematic representation of SRA-mRNAs and gene structure. **(A)** Three SRA1 cDNAs sharing a central core region but different in their 5′ and 3′ extremities have been originally characterized (Lanz *et al*., 1999). **(B)** The original noncoding SRA1 (AF092038) and genomic sequence (AC005214) have been aligned together with the three SRA1 sequences we cloned from normal breast cells. Vertical bars: initiating AUG (in exon 1) and stop codon (in exon 5). Gray stars: point mutations. Black star: point mutation followed by three bases insertion. The core is indicated in white and introns in black.

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(Fig. 1A), which shared a central core region, differed in their 5′ and 3′ terminal extremities, and defined a very peculiar coactivator, the Steroid Receptor RNA Activator (SRA; Lanz *et al*., 1999). Indeed, these transcripts, unable to code for any detectable protein *in vitro* or *in vivo*, specifically activated steroid receptors as RNA molecules, as opposed to all other previously described coregulators that act as proteins. It has now been shown that SRA1 RNAs modulate steroid receptor activity embedded in a ribonucleo–protein complex containing multiple proteins, such as the corepressor Sharp, the coactivator SRC-1, or the AF-1 specific activator p72/p68 protein (Lanz *et al*., 1999; Shi *et al*., 2001; Watanabe *et al*., 2001; Deblois and Giguere, 2003). The original SRA1 sequence (and the only human one put in Genbank by Lanz *et al*., #AF092038) is fully contained within five separated exon-like regions of the chromosome 5 genomic sequence (Fig. 1B). The core, which is necessary and sufficient for the noncoding RNAs to potentiate the activity of steroid receptors *in vitro* (Lanz *et al*., 1999) and to stimulate mammary cell growth *in vivo* (Lanz *et al*., 2003) encompasses exon-2 to a portion of exon 5. In 2000, we cloned from normal breast tissue three novel SRA1 mRNAs (AF293024, AF29325, and AF293026; Fig. 1B), mainly identical to the original SRA1 sequence but presenting an extended 5′ region. This region contained two ATG codons encoding the first methionines of putative 236/224 amino acid open reading frames. These coding variants, which contain an intact core region, are also expected to coactivate steroid receptors. These SRA1 variants as well as their corresponding SRA1 proteins (SRAP) have now been detected in human breast cancer cell lines, human breast tumor tissues, as well as in the skeletal muscle of various species (Leygue *et al*., 1999; Emberley *et al*., 2003; Chooniedass-Kothari *et al*., 2004, 2006). Even though SRAP function remains currently under investigation, our preliminary data have already shown that the expression of coding SRA1 RNA and its corresponding SRAP decreases the activity of estrogen receptor in MCF-7 breast cancer cells (Chooniedass-Kothari *et al*., 2006). Moreover, we also observed that ER positive breast cancer patients expressing detectable levels of SRAP in their primary tumor were less likely to die from the disease, suggesting that the protein itself could be a new prognostic marker for breast cancer (Chooniedass-Kothari *et al*., 2006). To our knowledge, SRA1 is the first, and so far only, molecule ever described to act at the RNA level and at the protein level. No data are therefore currently available on how such bi-faceted system could be regulated. Herein, we have addressed this question.

Using 5′-Rapid Amplification of cDNA Extremities (5′-RACE) and reverse-transcription polymerase chain reaction (RT-PCR) strategies, we investigated possible mechanisms regulating the balance between coding and noncoding SRA1 RNAs in breast cancer cells.

MATERIALS AND METHODS

In silico *prediction of transcription intitiation sites (TSS)*

Three online algorithms have been used to identify the putative transcription initiation sites of SRA1 transcripts. The input sequence, taken from the SRA1 genomic sequence (Genbank AC005214), corresponded to 1500 bp upstream of intron-1 + intron-1 + exon-2, totaling 1781 bp. Algorythms included DragonPF (http://research.i2r.a-star.edu.sg/promoter/promoter1_5/DPF.htm, expected TSS sensitivity 80%), McPromoter (http://genes.mit.edu/McPromoter.html, cutoff score −0.05) and NNPP2.2 (http://www.fruitfly.org/seq_tools/promoter.html, cutoff score 95%). CpG island prediction was performed using Cpgplot program (http://www.ebi.ac.uk/emboss/cpgplot/, observed/expected ratio >60, Percent G+C > 30, length > 50).

The database of transcription start sites (DBTSS, http://dbtss.hgc.jp/) is based on precise experimentally determined 5′-end clones, that is, sequencing of capped-mRNAs.

Human breast cancer cell lines

BT-20 (cat# HTB-19), MDA–MB-468 (cat# HTB-132), MDA-MB-231 (cat# HTB-26), MCF10A (cat#CRL-10317), ZR-75 (cat# CRL-1500), T47D (cat# HTB-133), and MCF7 (cat# HTB-22) breast cancer cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). T5 breast cancer cell line was kindly provided by Dr. LC. Murphy (Coutts *et al*., 1996). All the cells were grown up to 70% confluence, harvested, and cell pellets stored at −70°C, as previously described (Emberley *et al*., 2003). Total RNA and genomic DNA were extracted from cell pellets using Trizol™

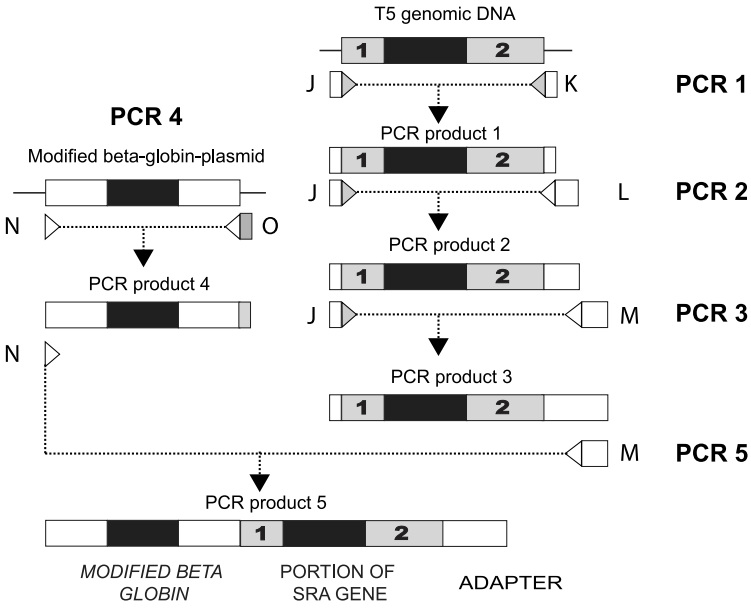

FIG. 2. Minigene construction. The insert part of the minigene has been constructed as described in the Materials and Methods section. Briefly, we first amplified (PCR 1) the genomic DNA from T5 cells using an upper primer (J) overlapping the 3′ end of the beta-globin construct and the 5′ end of the coding SRAP sequence, and a lower primer (K) containing the end of SRA1 exon 2. Two additional PCRs (PCR2 and PCR3), performed using J–L and J–M, respectively allowed the addition of the adapter region. The beta globin part of the construct was amplified (PCR 4) from a previously constructed plasmid (Dominski and Kole, 1991; Xie *et al*., 2005) using an upper beta globin specific primer (N) and a lower hybrid primer (O) containing a 3′-extremity specific for the end of beta-globin exon and a 5′-extremity corresponding to the beginning of SRAP coding region. The coamplification of the mixed products 3 and 4 allowed the production of a full insert subsequently cloned in the expression vector.

reagent (Gibco BRL, Grand Island, NY) according to the manufacturer's instructions.

5′-RACE

Total RNA (2 μg) from T5, MCF-7, and MDA–MB-468 was used as starting substrate to perform 5′-RACE using the SMART™ RACE cDNA amplification kit (Clontech, Mountain View, CA) following the manufacturer's instructions. The SRA1 specific lower primer used consisted in SRAL1 (sequence Table 1), annealing with the end of the core sequence and previously shown to effectively amplify SRA1 isoforms in breast cancer cells (Emberley *et al*., 2003).

RT-PCR, Triple-primer-PCR (TP-PCR)

One microgram of total RNA from the various cell lines was reverse transcribed in a final volume of 25 μl using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase and random hexamers and analyzed as previously described (Emberley *et al*., 2003). One microliter of reverse-transcription mixture was amplified in a final volume of 30 μl, in the presence of 60 mM Tris-HCl (pH 8.5), 15 mM $[NH_4]^2SO_4$, 1.5 mM $MgCl_2$, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dGTP, 0.2 mM dCTP, 4 ng/μl of each primer (pairs or three primers for RT-PCR and TP-PCR, respectively), 1 unit of *Pfu* DNA polymerase (Sratagene, La Jolla, CA) and 10 nM α-^{32}P dCTP. Each PCR consisted of a 5-min preincubation step at 94°C followed by 30

TABLE 1. SEQUENCE OF PRIMERS USED

Primer	*Sequence*	*Used in*
SRAL1	AGTCTGGGGAACCGAGGAT	5′ Race, Fig. 5
A	GCTGCCCCGCTGGCCAAGCGGA	Fig. 5
B	GCCAAGCGGAAGTGGAGAT	Fig. 5
C	GGAAGTGGAGATGGCGGAGCTG	Fig. 5
D	ATGGCGGAGCTGTACGTGAAGC	Fig. 5
E	TACGTGAAGCCGGGCAACAA	Fig. 5
F	TACGTGAAGCCGGGTGAGCG	Fig. 5
G	GCCAAGCGGAAGTGGAGAT	Fig. 6
H	CCCCAGTATAAGCTAACAGT	Fig. 6
I	GACGTCTTCCAATGCCTGTT	Fig. 6
J	GCCACACTGAGTGAGATGACGCGCTGCCCC	Fig. 2
K	GAGGAGAGGGTTGGGGATAGGCTTGCCCTCTGGGGGATCCATCCTGGGGTG	Fig. 2
L	GCCTTTGGCGTAGAATCGAGACCGAGGAGAGGGTTGGGGATAGGCTTGC	Fig. 2
M	TTATTTATTTAGTAGAATCGAGACCGAGGAGAGGGTTGGGGATAGGCTTGC-CTTTGGCGTAGAATCGAGAC	Fig. 2
N	GTGCACCTGACTCCTGAGGAGAA	Fig. 2
O	GGGGCAGCGCGTCATCTCACTCAAGTGTGGC	Fig. 2
P	GTGCACCTGACTCCTGAGGAGAA	Fig. 8
Q	CTCTGGGGGATCCATCCTGGGGTG	Fig. 8

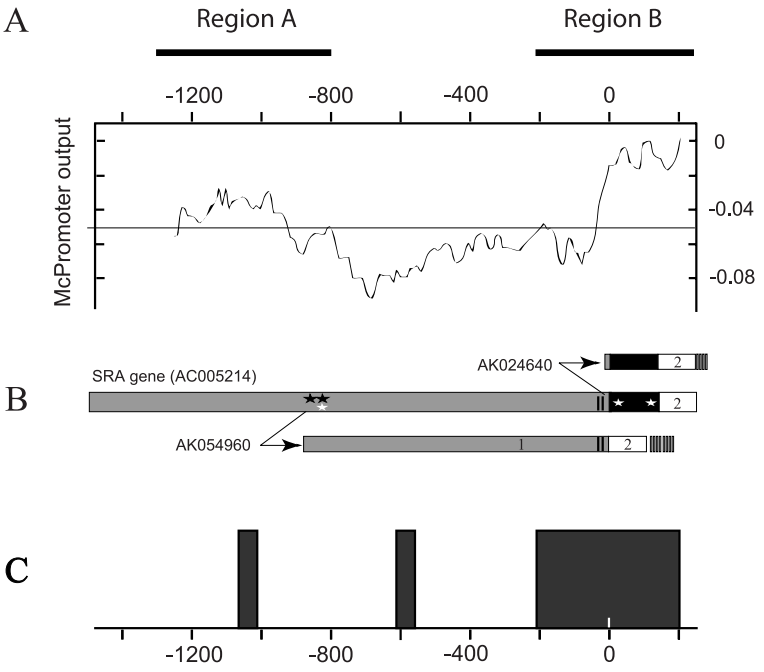

FIG. 3. Prediction of two promoter regions in the SRA1 gene. **(A)** One thousand seven hundred eighty-one base pairs corresponding to 1500 bp of exon 1, 155 bp of intron-1, and 126 bp of exon-2 were analyzed for putative transcription start sites (TSS) using three programs using cutoffs indicated in the Materials and Methods section. Sequence is numbered using the junction exon-1/intron-1 as 0. (Top panel) McPromoter prediction. The horizontal bar depicts the threshold above which the likelihood of TSS in the region considered is higher. Two promoter regions (A, B) are predicted. **(B)** The corresponding portion of the SRA1 gene sequence (AC005214) is depicted with exon-1 in gray, intron-1 in black, and exon-2 in white. Positions of the two ATG codons are indicated by vertical bars. Dragon promoter finder and NNPP2.2 predictions for TSS are indicated by white and black stars in the genomic sequence, respectively. The exact true initiation start site of two transcripts (AK024640 and AK054960) is indicated by arrows. **(C)** The positions of CpG islands identified using the CpG plot program on the same 1781 bp are indicated by dark boxes.

cycles of amplification (30 sec at 94°C, 30 sec at 60°C, and 30 sec at 72°C). The sequences of primers used are detailed in Table 1.

Radiolabeled PCR products were then separated on polyacrylamide gels as previously described (Leygue *et al*., 1999). Following electrophoresis, the gels were dried and exposed 30 min to a Molecular Imager™-FX Imaging screen (Bio-Rad, Hercules, CA). Exposed screen was then scanned using a Molecular Imager™-FX (Bio-Rad), which allows the subsequent quantification of each observed signal.

For further sequencing, PCR products were excised from the dry gel, reamplified in the absence of radioactivity, and subcloned using TOPO TA cloning® kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA).

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Minigene construction (Fig. 2)

One microgram of genomic DNA from T5 cells was amplified as described above in the absence of radiolabeled nucleotide and using primers J-K (PCR1; Fig. 2 and Table 1). The 384-bp PCR product 1 was separated on ethidium bromide-stained gel, cut out under UV, and purified using the Wizard® PCR preps DNA Purification System (Promega, Madison, WI) according to the manufacturer's instructions. This purified PCR product was then diluted (1/1000) and used as substrate for the subsequent PCR2 performed using J-L primers (Fig. 2 and Table 1). Similarly, the resulting PCR product 2 (407 bp) was reamplified using a J-M primer pair to generate the PCR product 3 (456 bp). A modified plasmid (kindly provided by Dr. Xie) containing human beta-globin exon sequences around a modified constitutive intron (Dominski and Kole, 1991; Xie *et al*., 2005) was then used as a target for amplification with primers N–O to obtain the PCR product 4 (414 bp). The two products, 3 and 4, overlapping in their 5′ and 3′ region, respectively, were purified, diluted, and mixed together. This mix was directly used as a template for the final amplification with N–M, allowing the generation of the PCR product 5 (840 bp). This latter product was subsequently purified and subcloned using the pcDNA4/HisMax TOPO® TA expression kit (Invitrogen) according to the supplier's instructions. Integrity of the final construction has been checked by sequencing (University Core DNA Services, Calgary, AB, Canada).

Transfection experiments

Transient transfections using the newly synthesized minigene were performed using lipofectamine™ reagent as described by the manufacturer. Twenty-four hours following transfection, cells were lysed and total RNA extracted, reverse transcribed, and amplified as described earlier using primers P-Q (Table 1).

Quantification of signals

TP-PCR signals from four independent experiments were quantified using a Molecular Imager™-FX (Bio-Rad) as previously described (Leygue *et al*., 1996). For each experiment and each cell line, the relative amount of fully spliced and intron-1 retained SRA1 was first expressed as the percentage of the total signal measured following amplification (individual percentage). The total signal corresponds to the sum of the signals of each band observed. The average percentage of intron-1 in T5 cells was then calculated (average T5) from the results of four independent experiments. For each experiment and each cell line (including T5), the individual percentage corresponding to intron 1 was then divided by this T5 average to obtained a normalized value. For each experiment and each cell line (including T5), these normalized percentages were subsequently averaged to obtain the global intron-1 expression (in arbitrary unit). The standard deviations were calculated. The differences between cell lines were tested using the Student's *t*-test (two

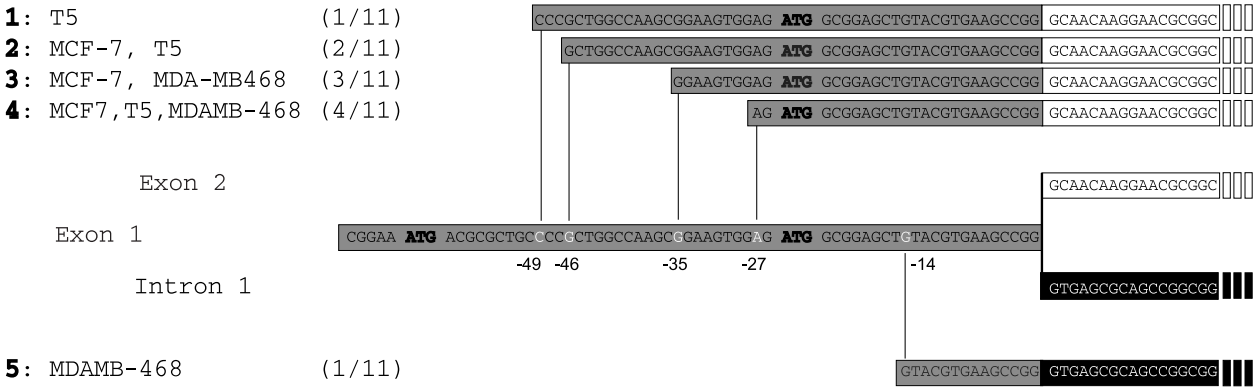


FIG. 4. Identification of putative transcription start sites located within the region B and used in breast cancer cell lines. Total RNA, extracted from T5, MCF-7, and MDA–MB-468 breast cancer cell lines, was analyzed by 5′-RACE as described in the Materials and Methods section. Eleven clones have been sequenced and five putative transcription start sites identified (1–5). For each site, the cells where the transcripts came from, the frequency of occurrence (x out of 11), the exact position on exon-1 sequence (corresponding nucleotide in white), as well as the 5′ sequence are indicated. Numbers are given relatively to the junction exon-1 (gray box)/intron-1 (black box). Exon-2 sequences are boxed in white. The two initiating ATG codon are bolded.

tails, equal variance assumed). A similar procedure was used to express the signal of each minigene PCR product in MDA–MB-468 versus T5 cells.

RESULTS

Putative and experimentally determined transcription SRA1 RNAs initiation sites

As outlined earlier in the text, the main difference between the original noncoding and the coding SRA1 isoforms laid within an extended exon 1 sequence (Fig. 1). This observation raised the possibility that these transcripts might therefore be initiated at distinct positions within the SRA1 promoter. We used a series of softwares predicting putative transcription initiation sites (Fig. 3) with cutoffs detailed in the Materials and Methods section. We analyzed 1781 bp covering exon-1, intron-1, and exon-2 (Fig. 3). For position reference, we used the junction between exon-1 and intron-1 as position 0. Two regions (Fig. 3A), region A (−1300/−800) and region B (−200/+200), are predicted by McPromoter to contain putative TSS. Such prediction is corroborated by both NNP2.2 and Dragon-promoter finder, which predict TSS at −839/−872 and −833/+32/+132, respectively (Fig. 3B). Interestingly, these two regions also contain CpG islands, often associated with genomic sequences highly involved in transcription initiation (Fig. 3C).

Only two SRA1 RNAs sequences are currently present in the database of transcription start sites (DBTSS), which contains only experimentally checked integral 5′ end cDNA sequences (Fig. 3B). The first sequence (AK054960), isolated from cerebellum, starts at position −883. It therefore contains the two ATG codons (position −61 and −25, respectively) and is able to code for the SRAP. The second (AK024640), cloned from endothelial cells of human coronary artery, is initiated at position −12. Not only it does not contain either of the methionine codons, but it also retains intron-1 sequences (155 bp long), which introduces a shift in the SRAP reading frame. This RNA is therefore unable to lead to the production of SRAP.

5′-RACE identification of transcription intitiation sites in three breast cancer cell lines

To interrogate the 5′-extremity of SRA1 transcripts present in breast cancer cells, total RNA was extracted from three breast cancer cell lines (MCF-7, T5, and MDA–MB-468) and analyzed by 5′-RACE as described in the Materials and Methods section. Eleven clones have been sequenced (three, three, and five clones from T5, MDA–MB-468, and MCF-7, respectively) and five possible initiation sites identified, clustered in a region located at the 3′ end of exon-1 (Fig. 4). Four of these sites (1–4, positions −49, −46, −35, and −27, respectively), corresponding to fully spliced RNAs (exon 1/exon-2 junction seen), are located upstream of the second ATG codon, suggesting that transcripts initiated in the region A can also be coding and fully spliced. Interestingly, one clone identified in MDA–MB-468, and starting downstream of the second ATG codon (site 5, position −14) corresponded to a messenger containing a full retention of intron 1, as seen for AK024640. That observation was of particular interest, as the retention of intron-1 makes such transcript unable to code for the SRAP but keeps it functional at the RNA level, as the core sequence (exon-2 to exon-5) is preserved.

Coding/noncoding SRA1 transcripts population in two breast cancer cell lines

To characterize the population of SRA1 transcripts detectable in breast cancer cells, a series of radiolabeled RT-PCR was performed using different upstream primers and a unique downstream primer recognizing the end of the core sequence. As shown in Figure 5A, the upper primers (A–F, position −54, −42, −35, −25, and −14) spanned the region surrounding the second ATG codon and are shown in the 5′-RACE experiments to overlap several TSS. Primer E was overlapping the exon-1/exon-2 junction, whereas primer F spanned the exon-1/intron-

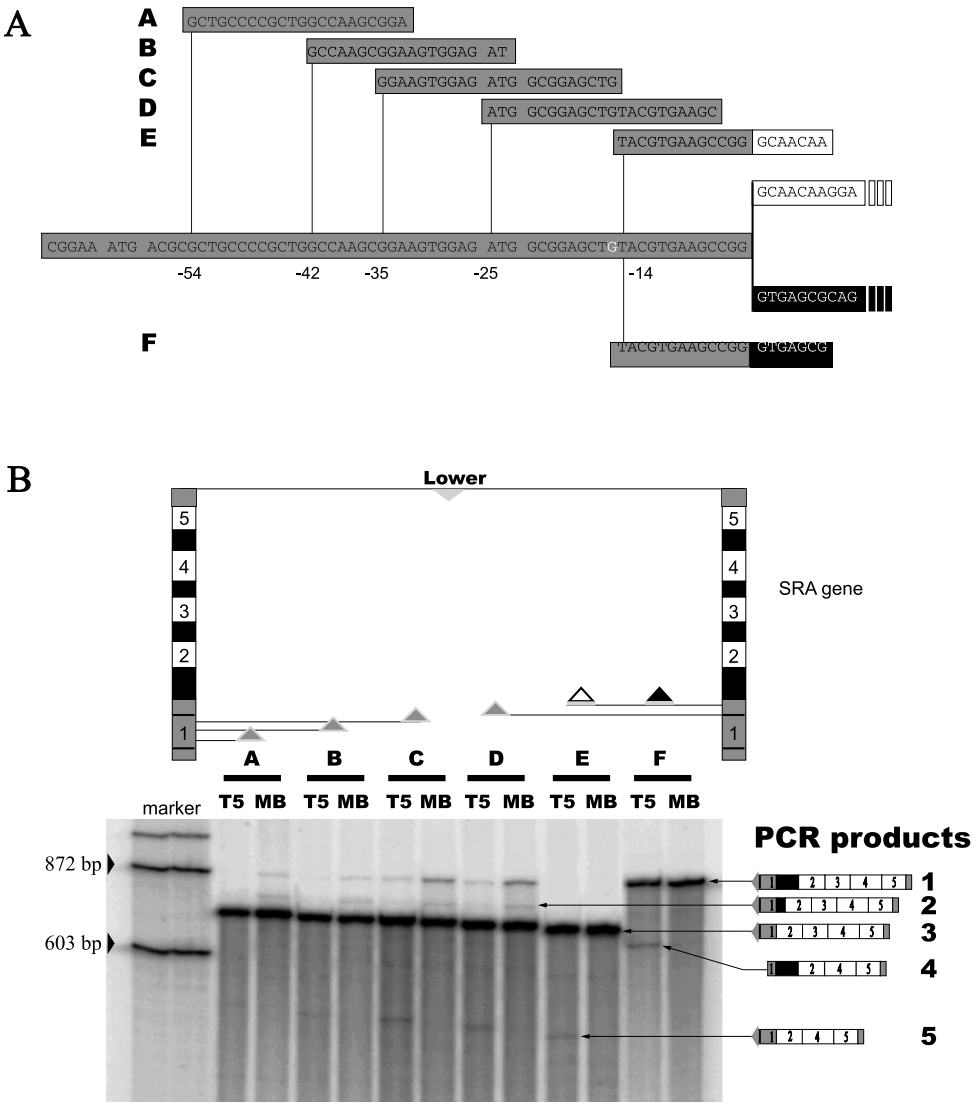


FIG. 5. Identification of SRA1 transcripts in T5 and MDA–MB-468 breast cancer cell lines. Total RNA, extracted from T5 and MDA–MB-468 (MB) breast cancer cell lines, was analyzed by RT-PCR and radiolabeled PCR products separated on acrylamide gels as described in the Materials and Methods section. **(A)** The sequences and exact positions of the upstream primers used are shown. Numbers are given relatively to the junction exon-1 (gray box)/intron-1 (black box). Exon-2 sequence is boxed in white. The two initiating ATG codon are bolded. The white G corresponds to the position of the transcription start site 5 corresponding to the intron-1 retained noncoding SRA1 RNA identified by 5′-RACE (Fig. 4). Note that primers E and F overlap the junction exon-1/exon-2 and exon-1/intron-1, respectively. **(B)** The upper panel depicts the position of the primers used. The lower panel shows the radiography of the gel. PCR products have been sequenced and are schematically depicted on the right.

1 boundary. Total RNA from two different cell lines (T5 and MDA–MB-468) was extracted and reverse-transcribed before performing PCR as described in the Materials and Methods section (Fig. 5B). The PCR products (1–5) indicated by arrows have been cloned and sequenced, and the corresponding RNA composition depicted at the bottom right of Figure 4B. Beside the expected fully spliced and coding SRA1 RNA (PCR product 3), four additional species were reproducibly observed in the cells analyzed.

The top PCR product (PCR product 1) corresponds to a full intron-1 retention and appears, relative to the normally spliced transcript (PCR product 3), more highly expressed in MDA–MB-468 than in T5 cells. It should be stressed that this transcript is still detectable when using primers A–C, which are all upstream of the original site 5 (-14, Fig. 4) shown to generate such a transcript.

An additional transcript (PCR product 2) containing only the 3′ part of intron-1 (60 last nucleotides, position +96 to +155), but otherwise normally spliced (all other introns removed), is also detectable in MDA–MB-468 cells and at a lower level in T5 cells. This transcript could act as coactivator as it contains a full core, but does not encode a SRAP. Indeed, the 60 inserted bases bring a premature stop codon to the SRAP open reading frame initiated at the ATG codons. As the fully intron-1 in-

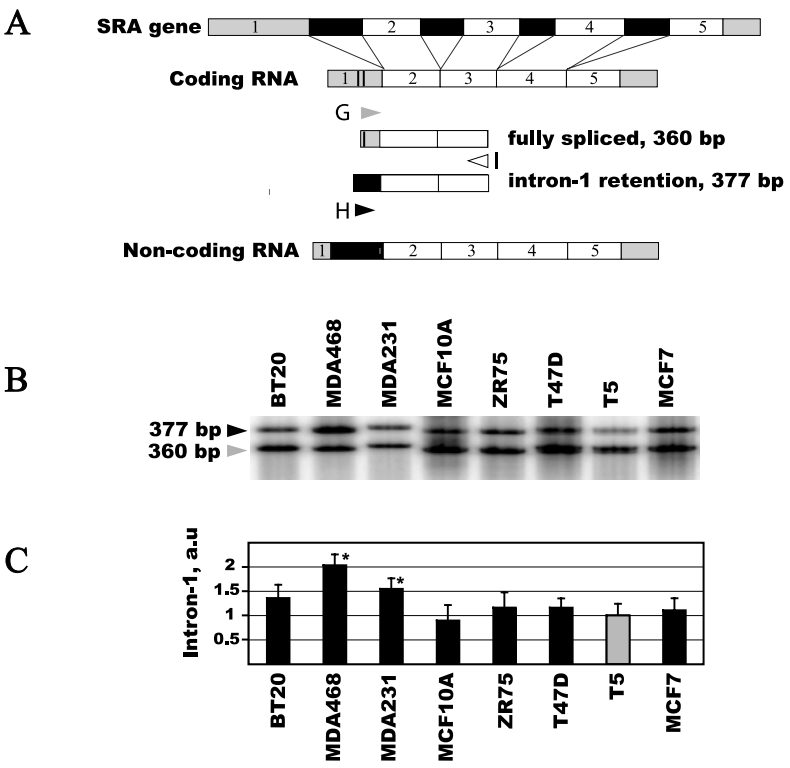


FIG. 6. TP-PCR amplification of coding and noncoding SRA1 mRNAs in breast cancer cells lines. **(A)** Principle: three primers are used during the PCR amplification. The lower primer (white, I) is common to the two sequences, whereas the upper G (gray) and H (black) primers are specific for exon-1 (gray) and intron-1 (black), respectively. Following PCR, the relative proportion of the signals obtained is proportional (but different) to the original relative amounts of the starting cDNAs (Leygue *et al.*, 1996). **(B)** Total RNA was extracted from BT-20, MDA–MB-468 (MBA468), MDA–MB-231 (MBA231), MCF-10A, ZR-75, T47D, T5, and MCF-7 cell lines, reverse-transcribed, and PCR amplified in the presence of P^{32}-dCTP using the three primers as described in the Materials and Methods section. PCR products were separated on acrylamide gel and visualized using a Molecular Imager™-FX. Bands migrating at 377 base pairs and 360 bp were sequenced and correspond to RNA containing intron-1 and fully spliced, respectively. **(C)** Signals from four experiments have been quantified as detailed in the Materials and Methods section. The average relative proportion of noncoding SRA1 RNA (intron-1 retained), expressed in an arbitrary unit, is graphed for each cell lines. Differences in relative intron-1 retained RNA expression between T5 (gray bar) and other cell lines (black bars) have been tested using the Student's *t*-test. Stars indicate a significant difference ($P < 0.05$). Standard deviations are shown.

serted transcript, this partial intron-1 inserted variant can also be detected using A–C primers. This simple observation suggests that SRA1 transcripts initiated upstream of the second ATG are alternatively spliced. This has been confirmed using other sets of primers (data not shown), and we have now submitted to Genbank a partial intron-1 SRA1 containing sequence starting at −250 (DQ286291). Interestingly, transcripts containing intron sequences (PCR product 4) or not (PCR product 5) and deleted in exon 3 were also identified. These transcripts are more highly expressed in T5 cells than in MDA–MB-468. They are noncoding, but are also not expected to act at the RNA level as they are missing a significant part of the core (202 nucleotides).

Altogether, these data confirmed the existence, within breast cancer cells, of alternatively spliced SRA1 transcripts, expressed at different levels between the two cell lines studied. The coexistence of transcripts fully spliced (not coding for SRAP) (PCR product 3) and of transcripts containing intron-1 sequences (not coding for the SRAP) (PCR products 1 and 2) is of particular interest. Indeed, it suggests that alternative splicing of this intron might be a possible mechanism used by breast cancer cells to regulate the balance between activator and repressor of estrogen receptor activity. To test for possible differences between cell lines in their relative amounts of coding (intron-1 normally spliced) and noncoding (total or partial intron-1 retained) mRNAs, we used a previously developed TP-PCR approach (Leygue *et al.*, 1996).

Differential relative splicing of SRA1 transcripts containing intron-1 sequence between breast cancer cell lines

As illustrated Figure 6A, the TP-PCR amplifies two PCR fragments corresponding to cDNAs sharing a common sequence (here exon 3) but different in their extremities (exon-1 versus intron-1 sequences). We have in the past shown that the proportion of obtained products was directly proportional to the ratio of the original cDNAs (Leygue *et al.*, 1996). The primer G spanned positions −42 to −24, and recognizes a fragment containing at least the second ATG, whereas primer H (+96 to +115) binds to both partial and full intron-1 retained RNA. Total RNA was extracted from a panel of breast cancer cell lines, reverse-transcribed and analyzed by TP-PCR as described in the Materials and Methods section. These cells were chosen to span a wide range of steroid receptors levels (e.g., MCF-7, ZR75, and T5 have a high level of estrogen receptor, T47D has high levels of progesterone receptor, and MDA–MB-231 is estrogen and progesterone receptor negative cells) and of invasion properties (MDA–MB-231 and MDA–MB-468 are highly invasive cells, whereas MCF-10A cells, coming from fibrocystic lesions, are often considered as "normal" (Miller *et al.*, 1993). PCR products, migrating around the expected sizes of 377 and 360 bases, were detectable in all cell lines tested. Sequencing of the products confirmed their identity as fragments corresponding to noncoding intron-1 and coding fully spliced SRA1 RNAs, respectively (data not shown). It should be stressed that a shift in size was observed

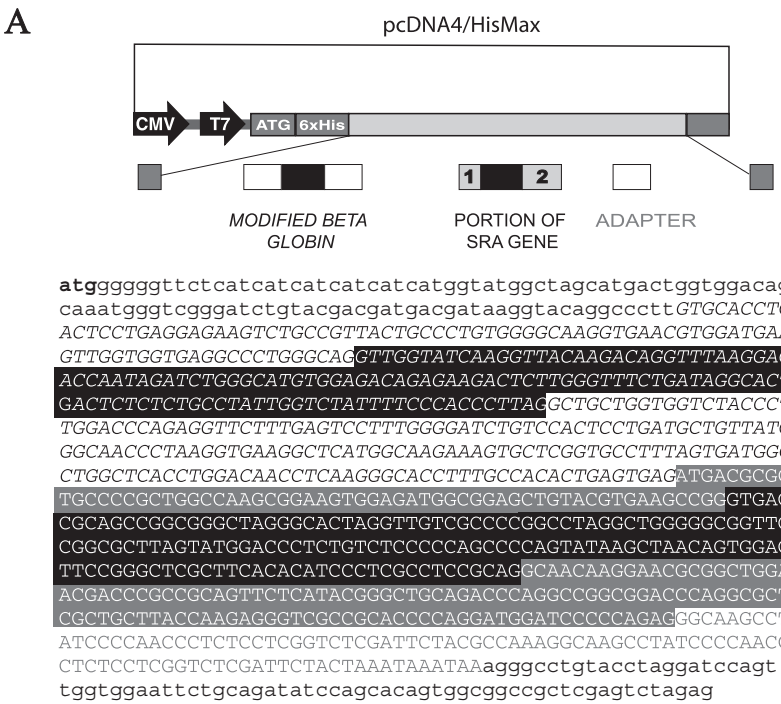
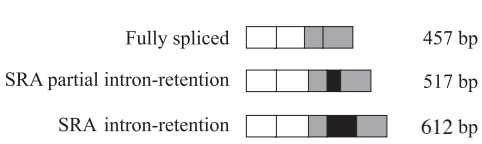


FIG. 7. Minigene design. **(A)** The minigene consisted in a pcDNA–4His/Max Topo backbone initiating a N-terminal His-tagged coding frame fused to a modified part of the beta-globin gene, SRA1 exon-1–intron-1–exon-2 sequences and an adapter. The exact sequence of the final product is shown. Lower case and capital letters correspond to plasmid and insert sequences, respectively. Italic and straight letters indicate beta-globin and SRA1 gene portion of the minigene, respectively. Intron sequences are boxed in black and SRA1 exons (1 and 2) are boxed in gray. **(B)** Expected PCR products obtained when using primers P and Q (see Table 1), recognizing the beginning of beta-globin exon and the end of SRA1 exon-2, respectively.



in some cell lines (e.g., slightly higher bands in MDA–MB-231 cells, lane 3, Fig. 6B). This shift results from the previously documented expression, in these cells, of the SRA1 isoform 3, containing a three-base insertion in exon 3, upstream of the lower primer used (see Fig. 1B; Emberley *et al*., 2003). Similarly, the known concomittent expression of isoforms 3 and 2 in T5 results in fully spliced and intron-1 retained corresponding bands to appear as a doublet (lane 7, Fig. 6B). The signals have been quantitated, and for each cell line, the intron-1 SRA1 RNA signal was expressed relative to its level in T5 cells (Fig. 6C). The relative proportion of SRA1 RNA containing intron-1 reproducibly varies from one cell type to another, illustrating that breast cancer cells differ in their balance of coding/noncoding mRNAs. Two cell lines, MDA–MB-468 and MDA–MB-231 expressed significant (Student's *t*-test, $P < 0.05$) higher relative amounts of SRA1 RNA retaining intron-1 than the T5 cells.

To establish whether these differences could result, in part, from a genomic diversity in this region, we extracted DNA from MDA–MB-468, MCF-10A, ZR-75, T47D, and T5 cells and sequenced genomic DNA from the first ATG codon to the middle of exon 2, as described in the Materials and Methods section. All sequences were identical (data not shown).

Minigene design and engineering

To further examine whether the primary genomic sequence in and around intron-1 was sufficient to lead to a differential splicing of this intron in different cell lines, we engineered a minigene depicted in Figure 7. The minigene contained a N-terminal His Tag fused to a modified part of the beta-globin gene and to SRA1 exon-1–intron-1–exon-2 sequences (Fig. 7A). The modified beta-globin gene portion contains a constitutively splice-able intron. Its correct removal in the transcripts subsequently analyzed demonstrated that RNAs still containing SRA1 intron-1 have been, even though only partially, processed by the splicing machinery. As shown Figure 7B, this construct has been designed to generate transcripts easily identifiable by RT-PCR using primers annealing with the beta-globin and SRA–exon-2 portions of the construct. The minigene has been constructed as described in the Materials and Methods section using a series of PCR amplifications, purifications, and dilutions of PCR products (Fig. 2).

Splicing of the minigene products in T5 and MDA–MB-468

The minigene has been transfected in T5 and MDA–MB-468 cells. Total RNA has been analyzed by RT-PCR 24 h after transfection, as detailed in the Materials and Methods section. The primers used (P-Q, Table 1) corresponded to the beginning of the beta-globin exon and the end of SRA1 exon 2. The results of three independent transfection experiments (experiments 1–3) are shown Figure 8. Two major products (full IR and fully spliced) were obtained, migrating at an apparent size of 612 and

457 bp (Fig. 8A). The sequencing of these fragments revealed that they corresponded to a RNA containing a full SRA1 intron-1 and to a fully spliced RNA, respectively (Fig. 8B). Two other products migrating at 517 bp (PIR) and 443 bp (AD), respectively, are also observable. PIR corresponded to the partial (60 last base pairs) intron-1 previously observed. Interestingly, SRA1 AD product is fully spliced, but is missing 14 bases at the end of exon-1, suggesting the possible use of a cryptic splicing site (Fig. 8B). It should be stressed that this splicing event introduces a premature stop codon in the SRAP reading frame, making the corresponding SRA1 transcript unable to encode the SRAP.

Following quantification, a significant higher relative expression of the SRA1 intron-1 retained mini-gene transcript product was observed in MDA–MB-468, when compared to T5 cells (Fig. 8C). As a direct result, the relative proportion of fully spliced transcript was significantly lower in MDA–MB-468 cells. No difference in the relative expression of the other products, PIR and AD, was observed.

DISCUSSION

The main difference between the originally isolated noncoding SRA1 RNA (Lanz *et al*., 1999) and the coding SRA1 isoforms we discovered, consisted in an extended exon 1 sequence (Emberley *et al*., 2003). We therefore first investigated the possibility that these coding and noncoding RNAs might originate from transcriptions initiated at different positions within the SRA1 gene promoter. Predictive algorithms indeed suggested the presence, upstream of the junction between exon-1/intron-1, of two regions (A and B) likely to house a series of transcription initiation sites. The cloning by others of two transcripts, fully capped and corresponding to these two regions, corroborated this assumption. The observation that the transcript initiated in the region A was coding, whereas the one originating from the region B was noncoding, also suggested that the balance between coding and noncoding SRA1 RNA could be controlled by an initiation of transcription in one region versus another. The fact that these transcripts originally came from two different tissues (cerebellum and artery) also raised the possibility that the alternative use of a given site might be tissue specific.

Using 5′-RACE, we herein identified five putative transcription initiation sites, clustered in the region B, located around the second ATG codon, and leading to the production of fully spliced coding SRA1 RNAs (sites 1–4) or noncoding intron-1 retained transcript (site 5). Such "sliding" of the exact initiation site is a typical feature of TATA-less promoter (Smale, 1997). It is here worthy to note that, whereas we obtained coding SRA1 in 10 out of the 11 clones we sequenced, Lanz *et al*. (2000) reported that they did not obtain any sequences containing initiating ATG codons when performing 5′RACE experiments. The simpliest explanation for such a discrepancy possibly lays within the fact that we used breast cancer cells, whereas the tissue origin of cells used by these authors is not specified.

Even though we have not used capped RNAs as a template to perform the 5′-RACE experiments, we are confident that the sites identified are true initition start sites. Indeed, the fact that we obtained exactly the same sites in different cell lines (sites 2–3–4, Fig. 3) reduces the possibility that we sequenced RNAs degradated exactly at the same position. Moreover, the site identified for the intron-1 retained RNA (site 5, position −12) is extremely close to the one reported for AK024640 (−14) in endothelial cells of human coronary artery. Altogether, these observations suggest that transcripts initiated in the region B in breast cancer cells can be either coding or noncoding.

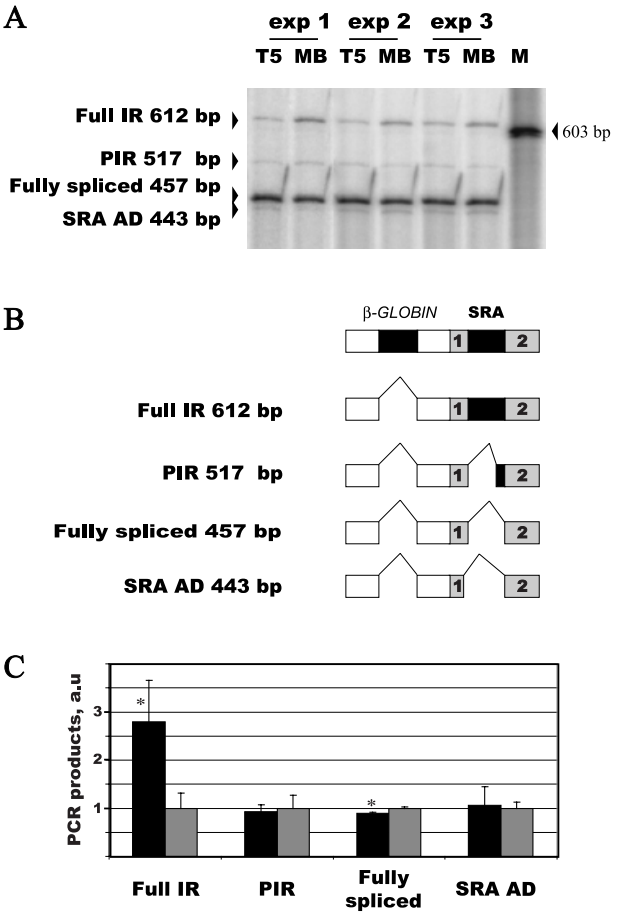


FIG. 8. Alternative splicing of minigene products in T5 and MDA–MB-468 cells. **(A)** Twenty-four hours following minigene transfection in T5 and MDA–MB-468 (MB) cells, total RNA was extracted, DNAse treated, reverse-transcribed and PCR amplified as described in the Materials and Methods section, using primers P and Q (Table 1). PCR products were separated on acrylamide gel and visualized using a Molecular Imager™-FX. The results of three independent experiments (exp. 1–3) are presented. Four bands, migrating at 612, 517, 457, and 443 bp were cloned and sequenced. **(B)** These bands corresponded to full SRA1 intron-1 retention (full IR), partial SRA1 intron-1 retention (PIR), fully spliced minigene transcript and a new SRA1 alternative SRA1 donor site (SRA1 AD) transcript, respectively. M: molecular weight marker, Φ×174 RF DNA/Hae III fragments. **(C)** For each band (full IR, PIR, fully spliced and SRA1 AD), signals have been quantified and expressed in arbitrary unit, as described in the Materials and Methods section. Differences in relative expression between T5 (gray bars) and MDA–MB-468 (black bars) have been tested using the Student's *t*-test. Stars indicate a significant difference ($P < 0.05$). Standard deviations are shown.

The SRA1 RNAs we previously isolated from breast cancer cells (Genbank # AF293024-26) were amplified by RT-PCR using a primer at position −250 (Emberley *et al*., 2003). Their transcription was therefore initiated upstream of region B we identified by 5′-RACE. Using the same upper primer, we were able to amplify SRA1 coding transcripts in all breast cancer cell lines we tested, including T5, MCF-7, and MDA–MB-468. This underlines the fact that transcripts likely initiated in the A region are also present in these cells (Emberley *et al*., 2003). We believe that we did not detect such transcripts in our 5′-RACE as a direct result of a preferential amplification of shorter cDNAs in the PCR reaction. The observation that intron-1 retaining transcripts can be amplified with the −250 primer (DQ286291) also confirms that the transcript initiated in region A can be alternatively spliced, and overall noncoding.

Altogether, our data show that in breast cancer cells, SRA1 transcripts can be initiated within two distinct regions (A and B), and that they can be alternatively spliced, independently of the site used. These data therefore strongly suggest that the generation of coding and noncoding SRA1 RNAs in breast cancer cells does not result from the initiation of the SRA1 transcripts at a particular site, but rather results from alternative splicing events involving intron-1 sequences.

We showed here that, indeed, coexpression of coding fully spliced and noncoding retaining intron-1 SRA1 RNAs is a common feature of breast cancer cell lines. We also demonstrated that cell lines differ in their relative levels of both RNA species. The observation that an artificial minigene generated RNA, containing exon-1, -intron-1, and exon-2 sequences is differentially spliced in these cell lines, and that the relative trend observed with endogenous SRAs is also seen with the artificial minigene product is of particular interest. Indeed, this suggests that the primary sequence of the transcript in this region, that is, exon-1/intron-1 and exon-2, is sufficient for the cell type specific differential intron-1 retention to occur.

The use by cells of alternative splicing of an intron to control the balance between coding and noncoding RNAs is certainly not a unique feature of the SRA1 gene. Indeed, intron retention is now considered as a relatively common event involving almost 15% of human gene products (Galante *et al*., 2004).

Alternative splicing has been shown to control major normal events such as sex determination or tuning of brain receptor sensitivity. Moreover, alteration of splicing has also been involved in many pathological situations including but not limited to skin diseases (Wessagowit *et al*., 2005) neurodegeneration (Lee and Irizarry, 2003; Gallo *et al*., 2005), and cancer (Hall and Russell, 2005; Kalnina *et al*., 2005).

In some cases, alternative splicing leads to the production of new sequences (Stetefeld and Ruegg, 2005), which can encode for extended or truncated proteins with novel or altered functional properties (Stamm *et al*., 2005). In other cases, alternative splicing results in the introduction of an early stop codon or a shift in the reading frame, leading to the generation of noncoding RNAs. In this latter situation, the noncoding RNA is often seen as a passive byproduct of an event, the major effect of which is usually thought to consist in the absence of protein. Since protein production is considered to be the functional outcome of most genes, the production of noncoding RNAs, as a result of differential splicing, is thought in this case to be a mechanism for gene silencing. However, the bivalent nature of SRA1, that is, functional RNA as well as functional protein, sheds a new light on alternative splicing. Indeed, this mechanism can now also be seen as a way to control the balance between two functional units, RNA as well as its coding counterpart.

Regulation of alternative splicing is a complex phenomenon that involves a plethora of different factors and signal sequences (Staley and Guthrie, 1998; Shin and Manley, 2004). It is now admitted that the full understanding of the molecular mechanisms involved in alternative splicing events could lead to the development of new therapies, targeting the production of particular spliced forms (Garcia-Blanco, 2005; Hagiwara, 2005; Wilton and Fletcher, 2005).

In this context, we are currently investigating the exact mechanisms participating in the differential splicing of intron-1 in breast cancer cells. Indeed, we believe that the characterization of the mechanisms involved will lead to the development of new strategies aiming at modifying the balance between coding and noncoding SRA1 RNAs. Ultimately, this will allow us to control the equilibrium between activator and repressor of estrogen receptor activity in breast cancer.

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Appendix 3:

The Steroid receptor RNA activator protein (SRAP) belongs to a new family of transcriptional repressors.

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Running Title: SRAP represses transcription

Abstract

The steroid receptor RNA activator was originally described as the first non-coding RNA able to co-activate the activity of steroid receptors. We have then demonstrated that this once thought non-coding RNA is in fact also able to encode for a stable SRA protein (SRAP). In order to further investigate into SRAP mechanisms of action, we have identified SRAP interacting proteins by mass spectrometric analysis of SRAP co-immunoprecipitated samples. Functional gene annotation classification revealed that several of these SRAP interacting proteins are involved in the modulation of transcription. We have determined trough protein arrays that SRAP is indeed able to directly interact with various transcription factors. Furthermore, we have established that SRAP is associated to chromatin in MCF-7 cells. In light of these observations, we examined the possible effect of SRAP recruitment on transcription using the potent GAL4-VP16 hybrid transcription activation system. We observed that SRA possesses a transcriptional repressive activity capable of inhibiting the GAL4-VP16 transcription activity. This SRAP transcriptional repressive potential is sensitive to trichostatin A (a HDAC inhibitor) treatment suggesting an involvement of HDACs in SRAP mechanism of action. In support of this hypothesis, we determined that SRAP is able to co-immunoprecipitate HDAC activity. Together our results indicate that similarly to SRA RNA, SRAP interacts with transcriptional regulators and is involved in the modulation of transcription. However unlike SRA RNA, SRAP is capable of negatively regulate transcription through the recruitment of HDAC activity.

Keywords: Steroid receptor RNA activator, SRA, SRAP, transcription.

Introduction

The steroid receptors family of proteins control vital physiological, developmental and metabolic processes by regulating gene expression in response to endocrine signaling[1]. While receptor activation initially plays a pivotal role, the transcriptional regulation of target genes is ultimately determined by the interactions between receptors and co-regulatory molecules [2] .These co-regulatory molecules can be divided into two broad functional categories, co-activators or co-repressors, which respectively stimulate or inhibit receptor activity. Research into the mechanisms of action of these regulatory proteins has shown that they interact with steroid receptors and regulate their transcriptional activity by allowing or preventing the recruitment of the transcriptional machinery and or by modulating the chromatin structure [3]. Steroid receptor co-regulatory proteins are currently under extensive investigation as they offer a new window of opportunity for the manipulation of signaling pathways, which could result in the development of novel therapeutic and diagnostic strategies for diseases that involve steroid receptors such as prostate cancer and breast cancer.

The steroid receptor RNA activator (SRA) is an exceptional member of the extended family of steroid receptor co-regulatory molecules. Indeed, SRA was initially characterized as a non-coding RNA molecule able to co-activate steroid receptors [4], while all other co-activators known today are protein molecules. SRA's co-activation action was at first thought to be limited specifically to steroid receptors. Subsequent studies have however shown that SRA RNA is in fact able to regulate the activity of other nuclear receptors such as the thyroid, Vitamin-D, retinoic acid and peroxisome proliferator activated receptor gamma [5-8] as well as other transcription factors such as the

myogenic differentiation antigen 1[9]. SRA was shown to contain a core RNA sequence necessary and sufficient to mediate steroid receptor activity [4]. Detailed analysis of the SRA RNA secondary structure revealed the presence of several motives within the core sequence needed for its function. Indeed mutations altering these secondary structures have been shown to affect SRA's co-activating properties [10]. Several proteins, interacting with SRA, have been proposed as mediator of its function. These include the coactivator/corepressor SHARP [11], SRC1 [4], and the AF-1-specific activator p72/p68 protein [12]. In addition, post-transcriptional modification such as pseudouridylation by pseudouridine synthases Pusp-1p and Pusp-3p have also been demonstrated to participate in the ability of SRA to modulate receptor activity [7, 13]. We have established that SRA RNA was differentially expressed in normal and in breast tumor tissue and suggested that SRA RNA could be involved in mechanisms underlying breast tumorigenesis and breast tumor progression [14].

Our recent findings have added an additional intricacy to the products of the *SRA1* gene by demonstrating that the once thought non-coding SRA RNA is actually also able to encode for a SRA protein we will refer to as SRAP[15, 16]. We have established that this protein is well conserved among chordates and, indeed, an alignment of SRAP sequences from different species reveals the presence of two extremely well conserved domains (Figure 1), likely involved in a functional conserved role.

Today, most studies on SRA only target its functional non-coding aspect and the exact function of SRAP remains largely unexplored. Herein, we explored the putative function of SRAP by first identifying its interacting proteins partners.

Results

Identification of SRAP-interacting Proteins.

To determine the identity of proteins interacting with SRAP, we immunoprecipitated SRAP-V5 from a previously described cell system consisting of MCF-7 cells over-expressing V5 tagged SRA protein (MCF-7 SRAP-V5 High.A) as detailed in the Materials and Methods section. Whole cell extracts supplemented with V5 peptides, to compete out the immunoprecipitation of SRAP-V5, were used as negative control to identify proteins non-specifically precipitated (Figure 2). As expected, V5 tagged SRAP was detected upon Western blot analysis of an aliquot of the precipitated sample but not in the negative control (Figure 2). Tandem mass spectrometric analysis of immunoprecipitated samples identified a total of 110 unique proteins. We disregarded 23 of these proteins as non-specifically precipitated since they were present in both the immunoprecipitated sample and negative control (Figure 2).

It is important to note that, SRAP was present among the remaining specifically precipitated 87 proteins. Using the functional gene classification software *(http://david.abcc.ncifcrf.gov/)*, we organized the proteins specifically associated with SRAP into distinct gene ontology functional categories, such as protein biosynthesis (table I), protein degradation (Table II), proteins involved in transport (Table III) and chaperones proteins (tables IV). A list of proteins known to possibly be non-specifically purified in affinity purification has been compiled at the NURSA site (http://www.nursa.org/files/Corip_Blacklist.pdf). We utilized this information to assemble an additional group of proteins that might possibly be precipitated non-specifically with SRAP (Table V). There were 32 remaining proteins (table VI) that could

not be classified in any of the categories mentioned above. In order to build a hypothetical SRAP interactome, we submitted the 32 proteins to ingenuity pathways analysis software (Figure 3). Interestingly, 17 out of the 31 proteins (excluding SRAP) are nuclear proteins. Several of these proteins have been shown to interact with each other. Furthermore 11 out of the 18 nuclear proteins are involved in transcription and transcription regulation.

SRAP interacts directly with transcription factors.

The mass spectrometry analysis of proteins immunoprecipitated with SRAP showed that several interacting proteins are involved in transcriptional regulation. We therefore investigated SRAP ability to directly associate to transcription factors using protein arrays spotted with recombinant transcription factors (Figure 4). We used recombinant SRAP protein to monitor protein protein interaction. Control arrays were carried out to identify false positives due to non-specific interactions between the antibodies and transcription factors. Only interactions appearing in the sample but not the control array were tabulated. Recombinant SRAP is shown to interact with 40 different transcription factors (Table VII). Interestingly, we have found that SRAP interacts with transcription factors with different binding affinities as assessed by the strength of the immuno-detected signals.

Endogenous and transfected SRAP are associated with chromatin in MCF-7 cells.

As SRAP interacts with transcription factors, we investigated whether SRAP could be associated to chromatin. SRAP V5 High.A cells were treated with formaldehyde and proteins cross-linked to chromatin were analyzed by Western blot using anti-SRAP, anti-Sp3 and anti GAPDH as described in the Material and Methods section. Both SRAP-V5

tagged and endogenous SRAP were found to be associated to chromatin (Figure 5). This association was not observed in the absence of cross-linking. As anticipated, both long (SP3L) and short (SP3M) SP3 isoforms (~ 100 kDa and ~60 kda respectively) known to be associated with chromatin, were also detected in the DNA bound protein fraction. On the other hand and as expected, GAPDH, (37 kDa) a mainly cytoplasmic protein, was present in the protein lysate but not associated with chromatin.

SRAP has intrinsic transcriptional repressive activity that is sensitive to TSA treatment.

To address the possible effect of the recruitment of SRAP by transcription factors, we constructed a chimera protein by fusing SRAP to the potent transcriptional activator GAL4-VP16. GAL4-VP16 is a hybrid molecule consisting of the yeast activator GAL-4 DNA binding domain fused to the transcription activation domain of the herpes simplex virus protein VP16 and was previously shown to efficiently activate the transcription of Gal4 reporter vectors in HeLa cells [17]. We transfected GAL4-VP16 or the chimeric GAL4-VP16-SRAP construct together with a GAL4-RE-luciferase reporter vector into HeLa cells as described in the Material and Methods section. The transcriptional activation in cells transfected with GAL4-VP16-SRAP was significantly lower than the activation observed in cells transfected with GAL4-VP16 (Figure 6). These results thus suggested that SRAP decreases GAL4-VP16 transcription activation potential. In order to exclude any possible influence of SRA RNA, we used a GAL4-VP16-SRA vector where the SRA cDNA contained silent mutations (SDM1 and SDM7) that have previously been shown to nullify SRA RNA co-activation function [10]. We found that similarly to the wild type SRAP, SRAP SDM1/7 was also able to repress GAL4-VP16

transactivation therefore excluding any functional involvement of the SRA RNA. Treatment with trichostatin A (a specific HDAC inhibitor) completely relieved the transcriptional repression caused by SRAP. As expected the fusion of SHP, a known transcriptional co-repressor, to the GAL4-VP16 also led to the repression of GAL4-V16 transactivation [18]. This SHP mediated repression is also TSA dependent as previously demonstrated [18]. Altogether, these results thus suggest that SRAP possesses a transcriptional repressive activity possibly involving HDACs.

SRAP recruits HDAC activity.

To determine if SRAP belongs to a complex containing HDAC activity, we immunoprecipitated SRAP-V5 from SRAP V5 High.A nuclear extracts and assessed whether the precipitated complex contained HDAC activity, as described in the Material and Method section. The SRAP-V5 immunoprecipitated sample contained significantly more HDAC activity than the control MCF-7 cells immunoprecipitated sample (Figure 7). Furthermore, the addition of V5 peptide to compete out SRAP-V5 precipitation brings back HDAC activity to levels comparable to the one seen in un-transfected MCF-7 control cells. These results therefore demonstrate that precipitation of SRAP-V5 results in specific co-immunoprecipitation of HDAC activity.

Discussion

Herein, we established that SRAP form complexes with several proteins including transcriptional regulators. We showed that SRAP can directly bind transcription factors and that its fusion to VP-16 decreased the activity of this strong transcriptional activator. We found that this inhibitory action involves HDAC activity and that SRAP is closely associated to chromatin.

To investigate its functional role, we opted for a strategy aimed at identifying proteins forming complexes with SRAP. As no antibodies immunoprecipitating SRAP were available when we initiated this study, we used a previously established cell system consisting of MCF-7 breast cancer cells stably expressing SRAP-V5-tagged protein [19]. It is important to stress that in these cells both exogenous and endogenous SRAP are expressed at similar levels [19]. It therefore provided a suitable system where the SRAP-V5 tagged protein was not drastically over-expressed. This reduced the possibility to identify artefactual protein partners as a result of an unphysiological amount of exogenous immunoprecipitated SRAP.

As expected in immunoprecipitated complexes from total cellular extracts, the majority of SRAP interacting proteins identified were “house keeping” proteins. Such common proteins partners are indeed anticipated to interact with most, if not all, proteins as they are involved in general protein metabolism such as biosynthesis, degradation, transport and chaperoning. In addition, we also identified several proteins that have been cited as possibly non-specifically purified in affinity purification. These include cytoskeletal proteins such as various actins, myosins, and tubulins as well as metabolic enzymes involved in gluconeogenesis and tRNA synthesis.

Among the 32 partners identified as forming complexes with SRAP (Table VI) figured proteins known to physically interact with each other. For example YWHAZ, which belongs to the 14-3-3 family of proteins mediating signal transduction by binding to phosphoserine-containing proteins, has been shown to directly interact with NPM1 (nucleophosmin) and nucleolin (NCL)[20]. Similarly, POL2RC and TAF15 are known to both belong to the same transcription pre-initiation complex [21]. This simple observation, not only confirms that several independent complexes have been specifically co-immunoprecipitated with SRAP, but also underlines the fact that SRAP is likely involved in several different cellular processes. This is further confirmed by the heterogeneity of partners identified, which included enzymes (such as MTHFD1, methylenetetrahydrofolate dehydrogenase NADP+ dependent 1), phosphatases (such as PPP1, protein phosphatase 1, catalytic subunit, alpha isoform), proteins involved in RNA metabolism (such as SYNCRIP, Synaptotagmin binding cytoplasmic RNA interacting protein) and transcription factors.

The most striking finding of our study was the fact that the majority of the remaining (32) proteins were nuclear and several of these have been shown to be involved in transcriptional regulation. Among these were MBD3 (Methyl-CPG binding domain protein 3, a member of the nucleosome remodeling and histone deacetylase complex (Nurd) [22], BAF57 (a core subunit of the SWI/SNF chromatin remodeling complex)[23] and YB-1 (Y-box binding protein, a transcription factor with multiple functions including co-repression and co-activation of other transcription factors) [24]. The high number of factors involved in transcriptional regulation among SRAP interacting proteins strongly suggested that SRAP might also be involved in the modulation of transcription.

In a concerted effort to generate a database of proteins interacting with co-factors [25], a list of SRAP interacting proteins was made available in January 2007 (http://www.nursa.org/datasets.cfm). These proteins were identified by co-immunoprecipitating SRAP associated proteins from nuclear Hela cell extracts using now commercially available anti-SRAP antibodies. Most of the SRAP interacting proteins identified here were different than the ones we identified. This disparity is most likely due to the difference in cell system as well as antibodies used for immunoprecipitation. Nonetheless, 13 out of 48 proteins identified in this study are also known to participate in transcriptional regulation. Included in these were several members of the PBAF subgroup of the SWI/SNF chromatin remodeling complex. Polybromo 1 (also known as BAF 180) and BAF 200, two subunits uniquely present in the PBAF subclass of SWI/SNF remodelers as well as BAF 170, a hallmark of many ligand dependent nuclear hormone receptor binding protein modules [26], were identified. While we did not identify these exact members of PBAF complex, we have however found another member of BRG-1 associated factors, namely BAF 57, to be associated with SRAP in our cell system. Interestingly, BAF 57 has also been shown to interact with these components of the PBAF subclass of remodelers [26].

Altogether, these data raised the possibility that SRAP is embedded in complexes regulating transcription events. The direct binding of SRAP with many different transcription factors, revealed by protein array, supports this hypothesis. Among the factors recognized figured steroid receptors (ER-beta, and GR). The direct binding of SRAP to steroid factors was expected. Indeed, Kawashima et al. showed that a partial rat SRAP sequence, consisting of the second conserved domain of SRAP (see Figure 1), was

able to directly interact with the androgen receptor [27]. In addition, we have verified that SRAP is able to directly interact with ER-alpha, ER-beta as well as PR by glutathione-S-transferase pull down assays (data not shown). SRAP is also directly binding to several other transcription factors including MEF2A, which was also found in SRAP complexes isolated by O'Malley's group. The high number of transcription factors recognized indicates that many different pathways might be sensitive to the presence or absence of SRAP. Interestingly, SRAP interacted strongly with factors such as ER-beta and HAND 1 but weakly with ATF-1 and apparently not with other factors such as DR-1. These results suggest that SRAP selectively associates with several transcriptional factors and is likely to have differential impact on the pathways involved. Further studies are needed to assess SRAP action on each individual transcription factors and consequently SRAP role in the implicated pathways.

The observation that SRAP interacts with transcriptional regulators and transcription factors as well as associates with chromatin led us to hypothesize that it might itself participate in regulating transcription events. In order to establish the effect of SRAP recruitement on transcription sites, we investigated the consequence of SRAP fusion to the transcriptional activator GAL4-VP16. We showed that SRAP fusion to VP-16 decreased the activity of this strong transcriptional activator. In order to exclude any influence of SRA RNA, we generated an additional GAL4-VP16- SRA construct that contained a set of silent mutations (SDM1/7) demonstrated to nullify SRA RNA function without altering the SRAP coding sequence[10]. Similarly to the wild type SRA, the SRA SDM1/7 also decreased VP-16 transcriptional activity suggesting that the transcriptional repressive activity of SRA was solely attributed to SRAP.

Furthermore we determined that the transcriptional repressive activity of SRAP was sensitive to trichostatin A, and that SRAP belongs to complexes containing HDAC activity. The association of SRAP to such complexes is not surprising, as some of the SRAP interacting partners identified by mass spectrometry have been shown to directly or indirectly interact with HDACs. Indeed, MBD3 has been shown to directly interact with HDAC 1 and HDAC2 and is a known component of the NurD complex known to contain both nucleosome remodeling as well as HDAC activities [28, 29]. It has been suggested that the NurD complex is targeted to specific promoters by transcription factors resulting in transcriptional repression [30]. YWHAZ, another SRAP interacting protein identified in our study was also shown to directly interact with HDAC 4 and regulate its cellular localization [31]. Altogether, these observations strongly suggest that SRAP might negatively regulate transcription through HDAC recruitment on targeted promoters.

The transcriptional repressive effect of SRAP on the GAL4-VP16 system corroborates our initial findings showing that MCF-7 cells overexpressing SRAP had a decreased sensitivity to estradiol treatment. While both SRA RNA and protein were expressed in this system, we had suggested that the observed repressive effects on estrogen receptor signaling were likely attributed to SRAP rather than SRA RNA, as this RNA is known to co-activate steroid receptor pathways [19].

A repressive role of SRAP is however in direct contrast with Kawashima et al. observations which led the authors to hypothesize that SRAP might be a steroid receptor co-activator [27, 32]. These authors have initially reported the cloning of a rat SRA mRNA encoding a protein starting at the third methionine codon of the rat SRA cDNA sequence (Figure 1). Interestingly, they have shown that this truncated SRA protein directly

interacted with the androgen receptor (AR) and increased its ligand dependent transcriptional activity. Mutation analysis confirmed the need for an open reading frame encoding a protein to be functional and led the authors to conclude that the SRA molecule acting as a co-activator was likely a protein, as opposed to an RNA molecule. A careful examination of the sequence used in these experiments revealed however that it started well within the core SRA sequence shown to be necessary for full co-activation by the SRA RNA. The observed absence of effect of SRA RNA might therefore result in their study from an important missing domain of the active core. The absence of the first conserved domain in the studied rat-SRAP also suggests that the effect observed might not correspond to the true SRAP function. In a subsequent study, the same group has shown that the full length SRAP was also able to co-activate AR. However, the SRA sequence used in transfection experiments contained the complete core sequence shown to be sufficient for SRA RNA function. The observed AR transcriptional activation might therefore, in these experiments, result from a combination of both SRA RNA and protein actions. SRAP might also be able to act as activator or repressor, depending upon the cellular context or the transcription factor involved. Further studies are needed to clarify these issues.

SRA is the first example of an increasingly complex bi-faceted molecule consisting of functional RNA and protein. In such a complex bi-faceted system, the possible concurrent actions of both entities present a challenge in determining their exact individual influences. There is therefore a crucial need for developing experimental approaches allowing the distinction between SRA RNA and SRAP functions in order to dissect further into their respective mechanism of action. The fact that SRAP interacts

with factors such as ER beta, GR or YB1, shown to be functionally and physically associated with SRA RNA [4, 11, 33, 34], strongly suggests that cross-talks exist between SRA and SRAP signaling. The involvement of both molecules in the same signal transduction pathways adds a further level of complexity to this system.

We propose that SRAP belongs to a new very well conserved family of proteins likely to act as a transcriptional repressors. SRA RNA has been previously found to act as a co-activator. This suggests that these two products of the *SRA1* gene are acting as two driving forces heading in two opposite directions.

Material and methods

Alignment of SRAP sequences: Putative SRAP sequence from Homo sapiens, Bos Taurus, Mus musculus, Rattus norvegicus, Sus scrofa, Equus caballus, Macaca mulata, Gallus gallus, Oryzias latipes, Occonrhyncus mykiss, Fugu rubripes, Silurana tropicalis, Xenopus laevis, Danio rerio, Ciona intestinalis and Ciona savignyi were obtained as described [15]. Additional sequences, Canis familiaris (CO618559, CO709664, TC18506), Pongo Pygmaeus (CAH93286), Ovis aries (CN823374), Salmo salar (CK898692, CK897849, CK885179) were processed similarly to get the corresponding SRAP protein sequences. All the putative SRA protein sequences were then aligned using the Multalin alignment tool (http://prodes.toulouse.inra.fr/multalin/).

Cell culture

Human breast cancer cell lines MCF-7 stably transfected with pCDNA 3.1 SRAP-V5 His (MCF-7 SRAP-V5 High.A) and MCF-7 stably transfected with pCDNA 3.1 -V5 His vector alone (MCF-7-Control) were previously described [15]. MCF-7 control, MCF-7 SRAP-V5 High.A and HeLa (ATCC) cells were cultured in DMEM (GIBCO, Grand Island, NY) medium supplemented with 5% fetal bovine serum (CANSERA, Rexdale, ON), penicillin (100 units/ml), streptomycin (100 µg/ml) (GIBCO, Grand Island, NY), and 0.3% glucose. Cells were grown in a 37°C humidified incubator with 5% CO_2.

Immunoprecipitation

MCF-7 SRAP-V5 High.A cells were lysed in 1X native buffer (50 mM NaPO4, 0.5 M NaCl pH 7.6) supplemented with protease inhibitor (Roche, Mannheim, Germany) through two successive freeze-thaw cycle using dry ice/ethanol and 42°C water baths

followed by 4 passages through an 18 gauge needle. The lysate was spun down and the supernatant was used for immunoprecipitation. Mouse IgG antibodies (Sigma, St Louis, MO) covalently linked to agarose beads were incubated with the lysate for 90 minutes at room temperature to pre-clarify the lysate. Following centrifugation, the supernatant was divided in two equivalent samples. One of the samples was incubated with anti-V5 antibodies (Sigma, St Louis, MO) covalently linked to agarose beads (binding capacity: 2.5 nmole/ml). The other sample was first supplemented with V5 peptide (350 times in excess of the antibodies binding capacity) and then incubated with anti-V5 antibodies covalently linked to agarose beads. Both samples were incubated on a rotary platform at room temperature for 90 minutes. Samples were then centrifuged and the beads were washed four times with 1X PBS. The immunoprecipitated proteins were eluted with 100 mM glycine-HCl (pH: 2.2). The eluates were neutralized with 0.5 M Tris. Samples were then either examined by mass spectrometric analysis or by Western blot analysis.

Western Blot analysis.

Immunoprecipitated samples were analyzed as previously described [19]. Mouse anti-V5 (Invitrogen Carlsbad, Ca), rabbit anti-SRAP 743 (Bethyl, Montgomery, TX), anti-SP3 (Santa Cruz, Santa Cruz, CA), mouse anti-GAPDH antibodies (Abcam, Cambridge, MA) were used at a dilution of 1/5000, 1/1000, 1/1000 and 1/1000 respectively. Secondary goat anti-mouse HRP (Sigma, St Louis, MO), or goat anti-rabbit HRP (Sigma, St Louis, MO), were used at a dilution of 1/3000.

Tandem mass spectrometric analysis of SRAP co-immunoprecipitated samples.

Immunoprecipitated samples were analyzed by mass spectrometry at the Manitoba Centre for Proteomics and System Biology (http://www.proteome.ca/). Briefly,

immunoprecipitated samples were eluted with 100 mM Glycine-HCl and neutralized with 0.5 M (final) Tris. Elution and neutralization buffers were removed by centrifuging the samples through filter tubes (centricon YM10) (Millipore Corporation, Bedford, MA) for 15 min at 5000 g. Samples were resuspended in 50 µl of 100 mM NH4HCO3, reduced in the presence of 5 µl of 100 mM dithiothreitol at 56 °C for 30 to 45 min and alkylated with 5 µl of 500 mM iodoacetamide at room temperature in the dark. Dithiothreitol and iodoacetamide were removed by centrifuging the filter tubes for 15 min at 13,000 rpm and the samples were resuspended in 50 mM NH4HCO3. Samples were digested by incubation with trypsin digestion buffer (50 mM NH4HCO3, 10 ng/µl bovine pancreatic trypsin (Calbiochem San Diego, CA)) overnight at 37 °C. The tryptic peptide fragments samples were lyophilized and resuspended in 0.1% TFA (trifluoroacetic acid) and analyzed by matrix assisted laser desorption ionization (MALDI) on a quadruple time of flight (QqTOF) mass spectrometry as previously described [35].

Database searches and protein identification.

The Global Proteome Machine (http://www.thegpm.org/) was used to process MS/MS data files and search the Human swissprot database to identify SRAP interacting proteins. We disregarded proteins that were common between the V5 peptide competition immunoprecipitated sample (negative control) and the immunoprecipitated sample. Using the functional gene classification tool softwares DAVID *(http://david.abcc.ncifcrf.gov/)*, PANTHER (http://www.pantherdb.org/) and the Ingenuity pathway analysis software (https://analysis.ingenuity.com/), we classified the proteins specifically associated with SRAP into distinct gene ontology functional categories and cellular localization.

Generation of recombinant SRAP

Recombinant SRAP proteins were generated by ProMab biotechnologies, Inc (Albany,CA). The SRA cDNA (AF293024) was cloned into pET-21a(+) bacterial expression vector. The resulting SRAP protein sequence was modified as follows. A N-terminal tag (MASMTGGQQMGRGSEF) was added prior to the first methionine which was changed to phenylalanine. The stop codon was omitted and a C terminal Histidine tag (KLAAALEHHHHHH) was added. This recombinant SRA protein was expressed in bacteria and purified via the histidine tag.

Panomics Array.

TF-protein array analysis (Panomics, Redwood City, CA) was performed as per manufacturers' instructions. Briefly, panomics TF protein arrays I, II, III and IV were incubated with 5 μg of recombinant SRAP protein diluted in 4 mL blocking buffer I for 2 hours at room temperature. A rabbit anti SRAP antibody targeted against amino acids 20 to 34 [19] diluted 1/1000 in 1X blocking buffer II was used as primary antibody. Secondary horseradish peroxidase-linked goat anti-rabbit antibodies (Bio-Rad, Hercules, CA) were then used. For each array, a duplicate blot was probed with primary and secondary antibodies alone in order to determine false positives (proteins that are non-specifically recognized by either the primary or secondary antibodies). Signals were analyzed by SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). Chemiluminescence signals were captured using the Fluor^{R}-S-Max MultiImager (Bio-Rad, Hercules, CA) and analyzed with the Quantity One system software (Bio-Rad, Hercules, CA). Dots appearing in the sample but not the control array were tabulated.

The strength of SRAP-protein interaction was consigned as strong (+++), moderate (++) or weak (+) depending on the intensity of the signals.

Isolation of DNA-associated Proteins by Formaldehyde Cross-linking

DNA associated proteins were isolated as previously described [36]. Briefly, MCF-7 SRAP-High.A cells were incubated with 1% formaldehyde for 10 min at room temperature. Reactions were quenched with the addition of 125 mM glycine in phosphate-buffered saline. As a control for non-specific precipitation by hydroxyapatite, MCF-7 SRAP-High.A cells were not cross-linked but incubated in phosphate-buffered saline alone for 10 minutes. After washing, cells were collected and resuspended in lysis buffer (5 M urea, 2 M guanidine hydrochloride, 2 M NaCl, and 0.2 M potassium phosphate buffer, pH 7.5). Sonication was carried out to solubilize chromatin and associated proteins, and cell lysates were collected following centrifugation. The cell lysates were then incubated with hydroxyapatite (Bio-Rad, Hercules, CA) (1 g of hydroxyapatite/80A_{260}). Samples were washed three times with ice-cold lysis buffer to remove proteins unbound to DNA. Cross-linking was reversed at 65 °C overnight, and following dialyze in water, DNA bound proteins were lyophilized. Proteins were resuspended in 1X SDS loading buffer and submitted to Western blot analysis. Anti-SRAP antibodies, anti-SP3 and anti-GAPDH antibody were used for immunodetection as described earlier.

Luciferase reporter gene assay.

The pGAL4-VP16 and pGAL4-VP16-SHP vectors were constructed as previously described [18]. The pGAL4-VP16-SRAP and pGAL4-VP16-SRAP SDM1/7 vectors were constructed as follows. The cDNA encoding SRAP was amplified by PCR using pCDNA3.1-V5-his containing either SRAP cDNA or SRAPSDM1/7 cDNA. Flanking 5’

EcoR1 site and 3' Hind III site were added during amplification. Following digestion the SRA or SRA SDM1/7 cDNA were inserted into pGAL4-VP16 previously digested with Ecor1 and Hind III.

HeLa cells were transiently transfected, using the cationic transfection reagent jetPEI^{TM} (Qbiogene inc. Carlsbad, CA) according to the manufacturer's protocol. Briefly, 0.5 μg of either pGAL4-VP16, pGAL4-VP16-SHP, pGAL4-VP16-SRAP or pGAL4-VP16-SRAP SDM1/7 were co-transfected with 0.5 μg of GAL4-RE- luciferase reporter (luciferase reporter vector containing a GAL-4 response element) plasmid and 0.1μg beta-galactosidase vector. The amount of plasmid vector was kept constant in all experiments. Trichostatin A (TSA, Sigma, St Louis, MO) or ethanol (vehicle) was added to the indicated concentration 16 hours before lysis. Cells were harvested 24 hours after transfection in a passive lysis buffer (Promega, Madison, WI). Aliquots were used for *beta*-galactosidase and luciferase activity assays. To measure *beta*-galactosidase activity, the lysate was mixed with a *beta*-galactosidase buffer (60 mM Na2HPO4, 40mM NaH2PO4, 10 mM KCl, 1 mM MgCl2, and 50 mM *beta*-mercaptoethanol) to which was added ONPG to a final concentration of 2 mM. The activity was given by absorption at the optical density of 415 nm. The luciferase activity was measured by the reaction of lysate with the luciferin solution (Promega, Madison, WI). Luciferase activities were measured as relative light units (RLU) on the LMax^{TM} microplate reader luminometer (Molecular Devices, Sunnyvale, CA). The luciferase activity was divided by the *beta*-galactosidase activity to account for transfection efficiency disparity between samples. The relative luciferase activity of cells transfected with pGAL4-VP16 was assigned as 1. The luciferase activities of cells transfected with the other vectors were expressed relative

to that of pGAL4-VP16. Results represented are the average of three independent experiments. Standard deviations were calculated and student's T test was used to assess significant differences between samples and control.

HDAC activity assay.

MCF-7 SRAP-V5 High.A and control MCF-7 were grown in normal conditions. Nuclear proteins were isolated using the nuclear extraction kit (Panomics, Redwood City, CA). MCF-7 SRAP-V5 High.A nuclear extract was divided in two equivalent samples. One of the MCF-7 SRAP-V5 High.A nuclear extract and the control MCF-7 cells nuclear extracts were incubated with anti-V5 antibodies covalently linked to agarose beads (binding capacity: 2.5 nmoles/ml). The other MCF-7 SRAP-V5 High.A nuclear extract was first supplemented with V5 peptide (350 times in excess of anti-V5 antibodies binding capacity) and then incubated with anti-V5 antibodies covalently linked to agarose beads. Immuprecipitation reactions were performed as mentioned earlier. HDAC activity was measured using the fluorometric HDAC assay kit (Abcam, Cambridge, MA) as indicated by the manufacturer, with the following modifications. After washing the beads three times with PBS, the samples were washed with 1 x HDAC assay buffer and then resuspended in 100 µl of HDAC assay buffer containing HDAC substrate, Boc-Lys(Ac)-pNA. Equal volume of anti-V5 antibodies covalently linked to agarose beads were used as blanks. In order to normalize for the differences in HDAC activities present in the MCF-7 SRAP-V5 High.A and control MCF-7 cell line, 5 µl of nuclear extracts from each cell lines were added to anti-V5 antibodies covalently linked to agarose beads, resuspended in 100 µl HDAC assay buffer containing HDAC substrate Boc-Lys(Ac)-pNA and assessed for HDAC activity along with the other samples. All samples were

incubated at 37 º C for 1 hour. Ten microlitres of lysine developer was then added and the samples were incubated for an additional half an hour at 37 º C. The samples were examined in a fluorescence plate reader (excitation =380 nm and emission= 450 nm). HDAC activities present in each immunoprecipitated samples were first normalized to the total HDAC activity present in the corresponding cell line nuclear extract as follows. Fluorescence readings of each immunoprecipitated samples were first divided by fluorescence readings of 5 µl of the corresponding cell line nuclear extract. The normalized fluorescence reading of the MCF-7 control immunoprecipitated sample was arbitrarily assigned as 1 for each experiment. The normalized fluorescence readings from the other two immnuprecipitated samples were then expressed relative to the activity of the immunoprecipitation control for each experiment and were qualified as relative HDAC activity. The relative HDAC activities were then averaged. Results are representative of four independent experiments. Standard deviations were calculated and significant differences between samples assessed using the Student's T test.

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Figure Legends.

Figure 1. Conservation of SRAP sequences. A) Alignment of Chordata SRAP sequences. The numbers indicated on top of the alignment correspond to amino acid sequence of the human SRAP protein encoded by SRA1 (Genbank #AF293024). Amino-acids conserved in all species are in red letters whereas those observed in 80%-100%, 60%-80% or less than 60% of all species are in orange, blue, and black, respectively. Within the consensus sequence, #, ! and + stand for D or E, I or V or M or L , and K or R, respectively. B) Schematic representation of human SRAP sequence. Red boxes show the two conserved regions. Positions are given relatively to the first methionine.

Figure 2. Strategy used to identify proteins forming specific complexes with SRAP. Total proteins from MCF-7 cells stably expressing SRAP-V5 were extracted, divided into two pools and subsequently immunoprecipitated with anti-V5 antibodies in the absence or presence of an excess of V5 peptide as described in Materials and Methods. Immunoprecipitated fractions (IP) or supernatant (Sup) were checked by Western blot using anti-V5 antibody. Total protein extracts (Tot) was used as positive control. Proteins contained in immunoprecipitated fractions were then sequenced by MS/MS, allowing the identification of proteins forming specific complexes with SRAP.

Figure 3. Cellular distribution of SRAP interacting protein. Proteins from Table VI were analyzed using the Ingenuity pathways analysis software. Proteins presented in diamond, triangle, and oval shapes are enzymes, phosphatases and transcriptional regulators, respectively. Others are depicted by circles. Proteins involved in RNA metabolism are in blue letters. Known protein-protein interactions are depicted by solid links.

Figure 4. SRAP directly interacts with transcription factors in vitro. Transcription factor protein array I was incubated with human recombinant SRAP (SRAP) or no protein (control) before immuno-detection with anti-SRAP antibodies as described in Materials and Methods. Signals seen on SRAP only (red boxes) as opposed to the one also seen on control (green boxes) reveals the specific interaction between SRAP and a given transcription factor.

Figure 5. Both endogenous SRAP and transfected SRAP-V5 associates to the chromatin. MCF-7 SRA-V5 High.A cells cross-linked with formaldehyde were lysed. DNA was precipitated from the extracts. Proteins associated to chromatin were eluted and analyzed by Western blot analysis. Chromatin associated protein fractions were analyzed for SRAP, SP3 and GAPDH detection. MCF-7 SRA-V5 High.A cell lysate was used as positive control. Chromatin associated proteins fraction from MCF-7 SRA-V5 High.A non treated cells was used as negative control.

Figure 6. SRAP possesses an intrinsic transcriptional repressive activity sensitive to TSA treatment. Hela cells were co-transfected with pGALVP16 (control), pGALVP16-SHP, pGALVP16-SRAP, or pGALVP16-SRAP SDM1/7 together with gal4-luciferase

reporter vector, and corresponding luciferase activity was measured, following cell lysis, as described in Materials and Methods. Luciferase activity is expressed relative to the activity in cells transfected with pGALVP16 (control) alone. Bars represent standard deviation.

Figure 7. Immunoprecipitation of SRAP-V5 from MCF-7cells stably expressing SRAP-V5 results in precipitation of HDAC activity. Cell extracts from MCF-7 cells previously stably transfected with control vector (MCF-7c) or encoding V5 tagged SRAP (MCF-7 SRAP-V5) were immunoprecipitated with anti-V5 antibodies (IP) and HDAC activity measured and expressed in arbitrary unit as detailed in the Materials and Methods section. As additional control, immunoprecipiation of MCF-7 SRAP-V5 was performed in parallel with competitive V5 peptide (IP + V5).

Figure 1 to 7: please see attached PDF file.

Table I: SRAP interacting proteins involved in protein biosynthesis.

Symbol	Name
EEF2	Eukaryotic translation elongation factor 2
EIF3S2	Eukaryotic translation initiation factor 3, subunit 2 beta, 36 kDa
EIF3S4	Eukaryotic translation initiation factor 3, subunit 4 delta, 44 kDa
EIF4A1	Eukaryotic translation initiation factor 4A, isoform 1
EIF4G1	Eukaryotic translation initiation factor 4 gamma,1
RPL6	Ribosomal protein L6
RPL7	Ribosomal protein L7
RPL8	Ribosomal protein L8
RPL14	Ribosomal protein L14
RPL18	Ribosomal protein L18
RPL23A	Ribosomal protein L23A
RPL24	Ribosomal protein L24
RPL27	Ribosomal protein L27
RPL31	Ribosomal protein L31
RPL32	Ribosomal protein L32
RPL35	Ribosomal protein L35
RPS10	Ribosomal protein S10
RPS14	Ribosomal protein S14
RPS16	Ribosomal protein S16
RPS18	Ribosomal protein S18
RPS2	Ribosomal protein S2
RPS26E	Ribosomal protein S26E

Table II: SRAP interacting proteins involved in protein degradation.

Symbol	Name
CACYBP	Calcyclin binding protein
PSMA2	Proteosome 26S subunit,ATPase, alpha type2
PSMC1	Proteosome 26S subunit,ATPase,1
PSMC5	Proteosome 26S subunit, non-ATPase,5
PSMD2	Proteosome 26S subunit, non-ATPase, 2
PSMD11	Proteosome 26S subunit, non-ATPase,11
PSMD12	Proteosome 26S subunit, non-ATPase,12
PSMD14	Proteosome 26S subunit, non-ATPase,14

Table III: SRAP interacting proteins involved in protein transport

Symbol	Name
ARF1	ADP-Ribosylation factor 1
ARCN1	Archain1
COPE	Coatomer protein complex, subunit epsilon
SEC13L1	SEC 13 like 1

Table IV: SRAP interacting chaperone proteins.

Symbol	Name
CCT3	Chaperonin containing TCP1, subunit 3 (gamma)
CCT4	Chaperonin containing TCP1, subunit 4 (delta)
CCT5	Chaperonin containing TCP1, subunit 5 (epsilon)
CCT8	Chaperonin containing TCP1, subunit 8 (theta)
CCT6A	Chaperonin containing TCP1, subunit 6A
HSPA8	Heat shock 70 kDa protein 8
HSPA9B	Heat shock 70 kDa protein 9B
HSPB1	Heat shock 27 kDa protein 1
HSP90AA1	Heat shock protein 90 kDa alpha, class A member 1
HSP90B1	Heat shock protein 90 kDa beta, member 1

Table V: Protein possibly non-specifically purified.

Symbol	Name
ACTB	Actin beta
ACTC	Actin alpha
ACTG2	Actin alpha 2
MLC1SA	Myosin ligth chain 1 slow A
MYL6	Myosin Light polypeptide 6, Alkali, smooth muscle and non-muscle
LMNA	Lamin A/\C
TUBB2	Tubulin beta 2C
GAPDH	Glyceraldehyde 3 phosphate dehydrogenase
G6PD	Glucose 6 phosphate dehydrogenase
LDHA	Lactate dehydrogenase A
EPRS	Glutamyl-prolyl- TRNA- synthethase

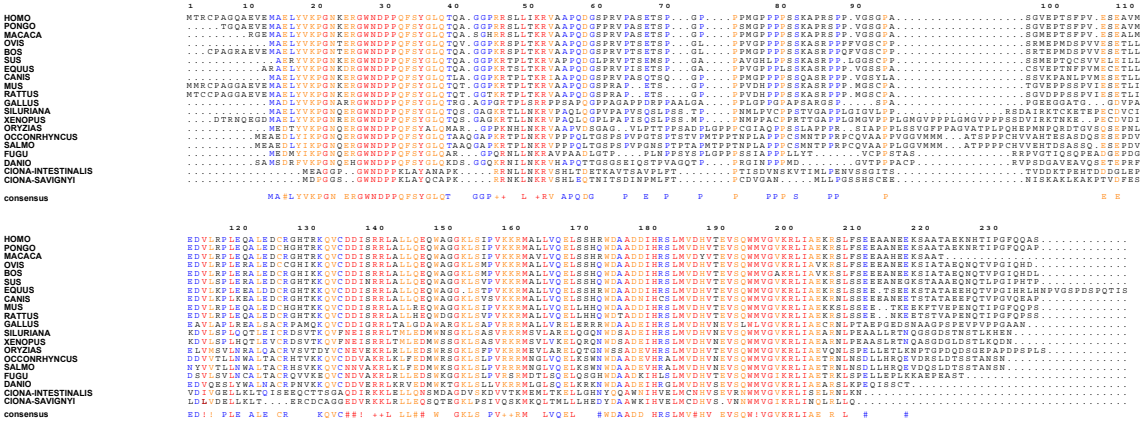
Table VI: SRAP interacting proteins

Symbol	Name
C14ORF166	Chromosome 14 open reading frame 166
CALM2	Calmodulin 1
CALR	Calreticulin
CNOT7	CCR4-NOT transcription complex
CSDA	Cold shock domain protein A
FASN	Fatty acid synthase
GNB1L	Gaunine nucleotide binding protein, beta, polypeptide 1 like
GOLGA2	Golgi autoantigen, golgin subfamily A,2
HIST1H1B	Histone 1, H1B
HIST1H1C	Histone 1, H1C
HIST1H4B	Histone 4, family 2
HIST2H2AA3	Histone 2, H2AA3
ILF3	Interleukin enhancer binding factor 3, 90 kDa
KHDRBS1	KH domain containing, RNA binding, signal transduction associated 1
KIAA0552	
MARCKS	Myristolated alanine-rich protein kinase C substrate
MBD3	Methyl-CPG binding domain protein 3
MTHFD1	Methyenetetrahydrofolate dehydrogenase (NADP+ dependent) 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase
NCL	Nucleolin
NPM1	Nucleophosmin
P4HB	Procollagen-proline, 2-oxoglutarate 4-dioxygenase
POLR2C	Polymerase (RNA) II (DNA directed) polypeptide C, 33 kDa
PPP1CA	Protein phosphatase 1, catalytic subunit, alpha isoform
PRDX6	Peroxiredoxin 6
PRKCSH	Protein Kinase C substrate 80 K-H
SMARCE1	SWI/SNF related, matrix associated, actin dependent regulator of chromosome
SRA	Steroid receptor RNA activator
SYNCRIP	Synaptotagmin binding, cytoplasmic RNA interacting protein
TAF15	TAF15 RNA polymerase II, TATA box binding protein (TBP)-associated factor 15
TSEN34	TRNA splicing endonuclease 34 homolog (S. Cervisiae)
YBX1	Y Box binding protein 1
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein

Table VII: Transcription factors interacting with SRAP

Transcription factor	Interaction	Protein description
ATF1	+	Activating Transcription Factor 1
ATF2	+	Activating Transcription Factor 2
ATF4	+	Activating Transcription Factor 4
BLZF1	+	Basic leucine zipper nuclear factor 1
CART1	+	Cartilage paired-class homeoprotien 1
CREB1	+	cAMP responsive element binding protein 1
DDIT3	+	DNA damage inducible transcript 3
DMTF1	++	Cyclin D binding myb-like transcription factor 1
E2F3	++	E2F transcription factor 3
EGR2	+	Early growth response 2
ESR2	+++	Estrogen receptor 2
ERRg	+	Estrogen related receptor gamma
ETS1	+	V-ets erythroblastosis virus E26 oncogene homologue 1
ETS2	+	V-ets erythroblastosis virus E26 oncogene homologue 2
FOS	+	V-fos FBG murine osteosarcoma viral oncogene homologue
FOSB	+	FBG murine osteosarcoma viral oncogene homologue B
FOSL2	+	Fos-like antigen 2
GATA1	+	GATA binding protein 1
GTF2H2	++	General transcription factor IIH, polypeptide 2, 44 kDa
HAND1	+++	Heart and neural crest derivatives expressed 1
HAND2	++	Heart and neural crest derivatives expressed 2
HNF4G	+	Hepatocyte nuclear factor 4 gamma
IRF1	+	Interferon regulatory factor 1
JUN	+	V-jun sarcoma virus 17 oncogene homologue
JUN B	+	JUNB proto-oncogene
LDB1	++	LIM domain binding 1
LHX2	++	LIM homeobox 2
MAX	+	MAX protein
MECP2	++	Methyl cpg cinding protein 2
MEF2A	+	MEF2A: MADS box transcription enhancer factor 2, polypeptide A (myocyte enhancer factor 2A)
NR3C1	+	Glucocorticoid receptor
PPARg1	++	Peroxisome proliferative activated receptor gamma
PTTG1	+	Pituitary tumor transforming 1
PTTG2	+	Pituitary tumor transforming 2
RYBP	++	RING and YY1 binding protein
SIX2	+	Sine oculis homeobox homologue 2
SP3	+	Sp3 transcription factor
TCEA1	+	Transcription elongation factor A,1
TFE3	+	Transcription factor binding to IGHM enhancer 3
YY1	++	YY1 transcription factor

A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MACACA
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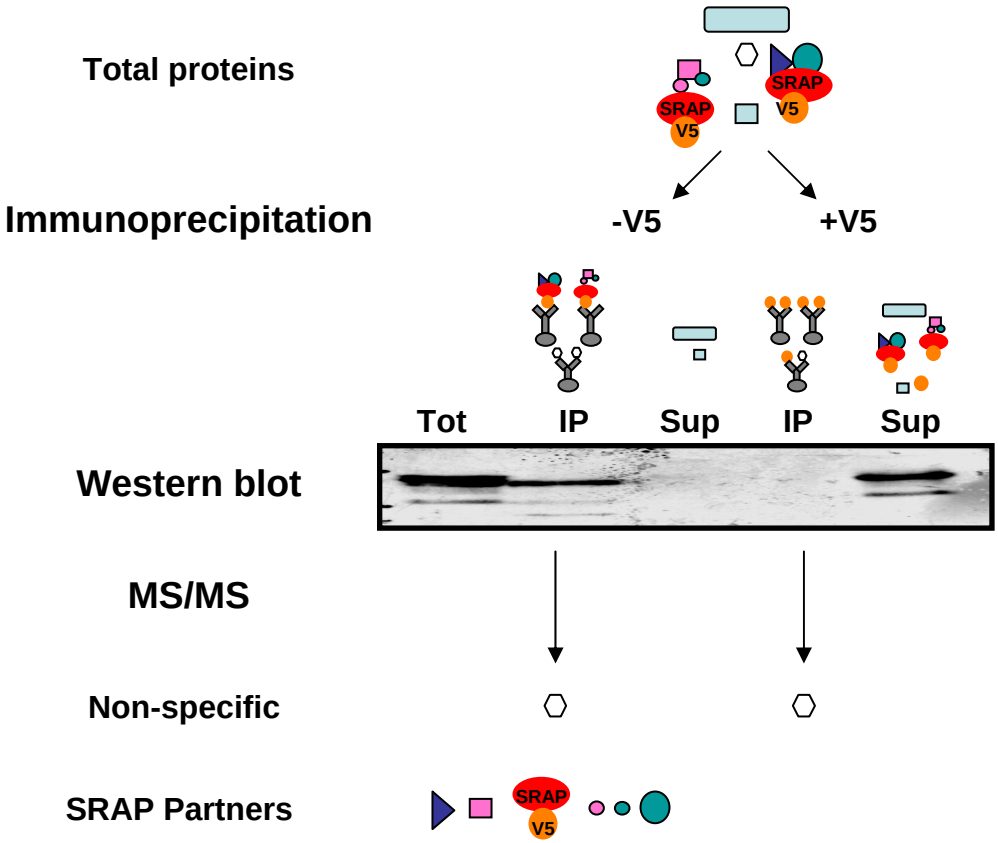
ORYZIAS
OCCONRHYNCUS
SALMO
FUGU
DANIO
CIONA-INTESTINALIS
CIONA-SAVIGNYI
consensus

B


N-
-C
15
52
135
204

Fig 1


Total proteins
SRAP
V5
SRAP
V5
Immunoprecipitation
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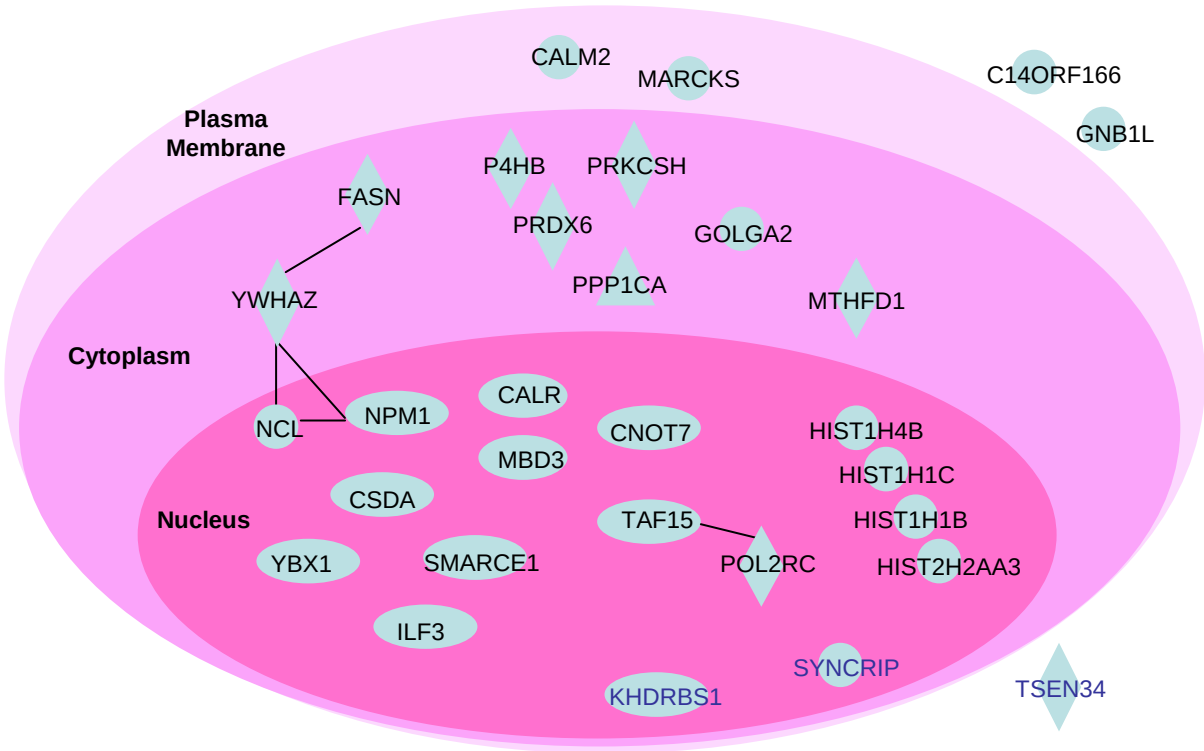
+V5
Tot
IP
Sup
IP
Sup
Western blot
MS/MS
Non-specific
SRAP Partners
SRAP
V5

Fig 2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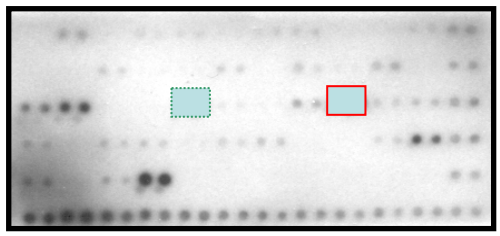
MARCKS
C14ORF166
GNB1L
Plasma Membrane
P4HB
PRKCSH
FASN
PRDX6
GOLGA2
PPP1CA
YWHAZ
MTHFD1
Cytoplasm
CALR
NCL
NPM1
CNOT7
HIST1H4B
MBD3
HIST1H1C
CSDA
Nucleus
TAF15
HIST1H1B
YBX1
SMARCE1
POL2RC
HIST2H2AA3
ILF3
SYNCRIP
KHDRBS1
TSEN34

Fig 3

Array

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	AES		AP2A		ASH2L		ATF1		ATF2		ATF3		ATF4		BLZF1		BTG2		C/EBPa		CART1		POS	
B	CBFB		CDX2		CREB1		CREBL2		CERM		CRSP9		ELK		DDIT3		DLX4		DMTF1		DR1		POS	
C	E2F3		E2F4		E2F5		E2F6		EGR1		EGR2		EGR4		ER-a		ER-b		ERRg		ETS1		POS	
D	ETS2		F2RL1		FOS		FOSB		FOSL1		FOSL2		GATA1		GCNF		GMEB1		GR		GTF2B		POS	
E	GTF2H2		GTF2I		GTF3C5		HAND1																POS	
F	POS		POS		POS		POS		POS		POS		POS		POS		POS		POS		POS		POS	

SRAP



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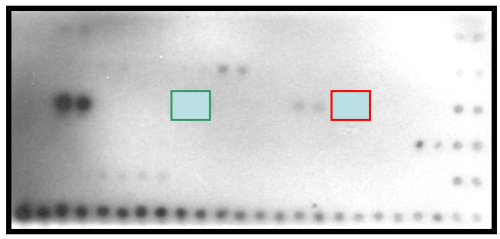
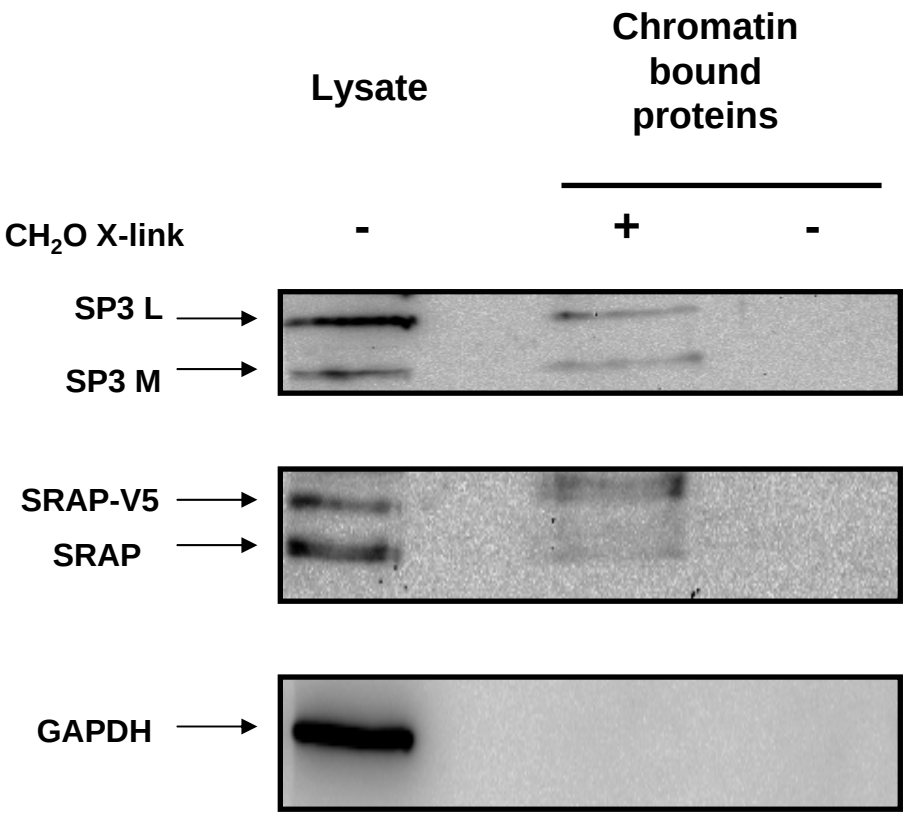


Fig 4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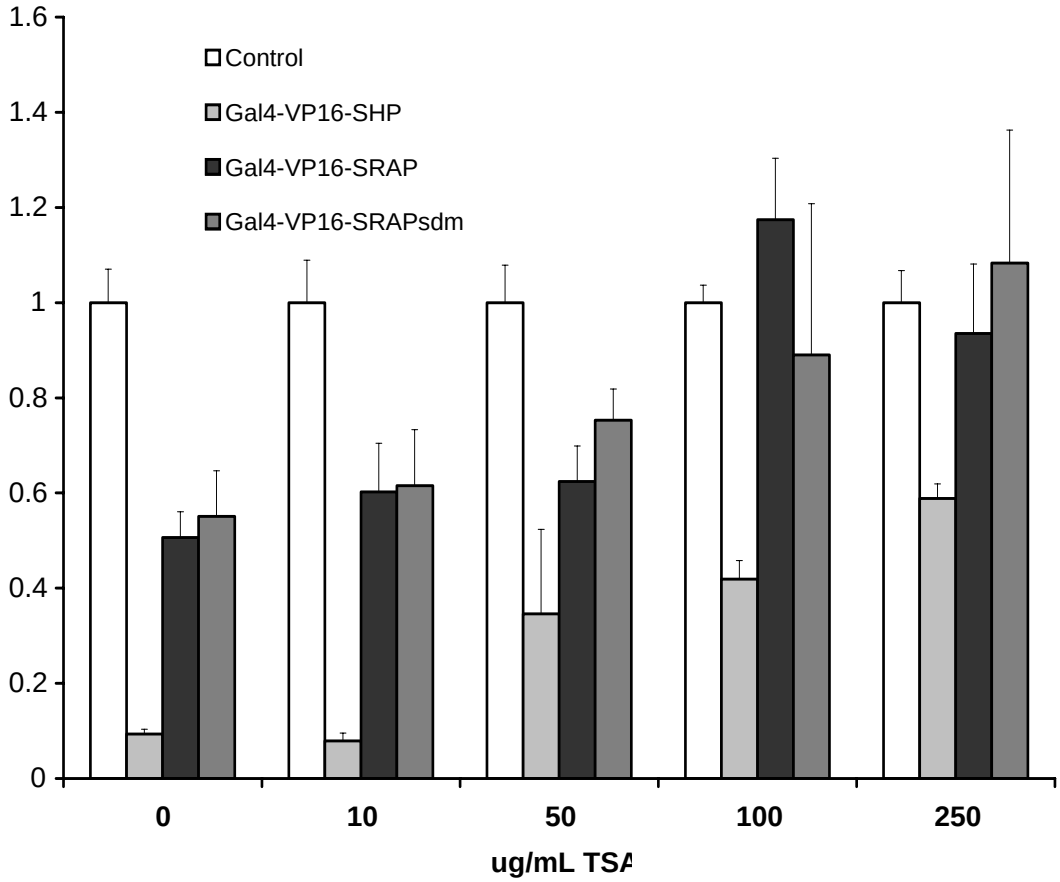
Chromatin bound proteins
CH2O X-link
-
+
-
SP3 L
SP3 M
SRAP-V5
SRAP
GAPDH

Fig 5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1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
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Gal4-VP16-SRAP
Gal4-VP16-SRAPsdm
0
10
50
100
250
ug/mL TSA

Fig 6

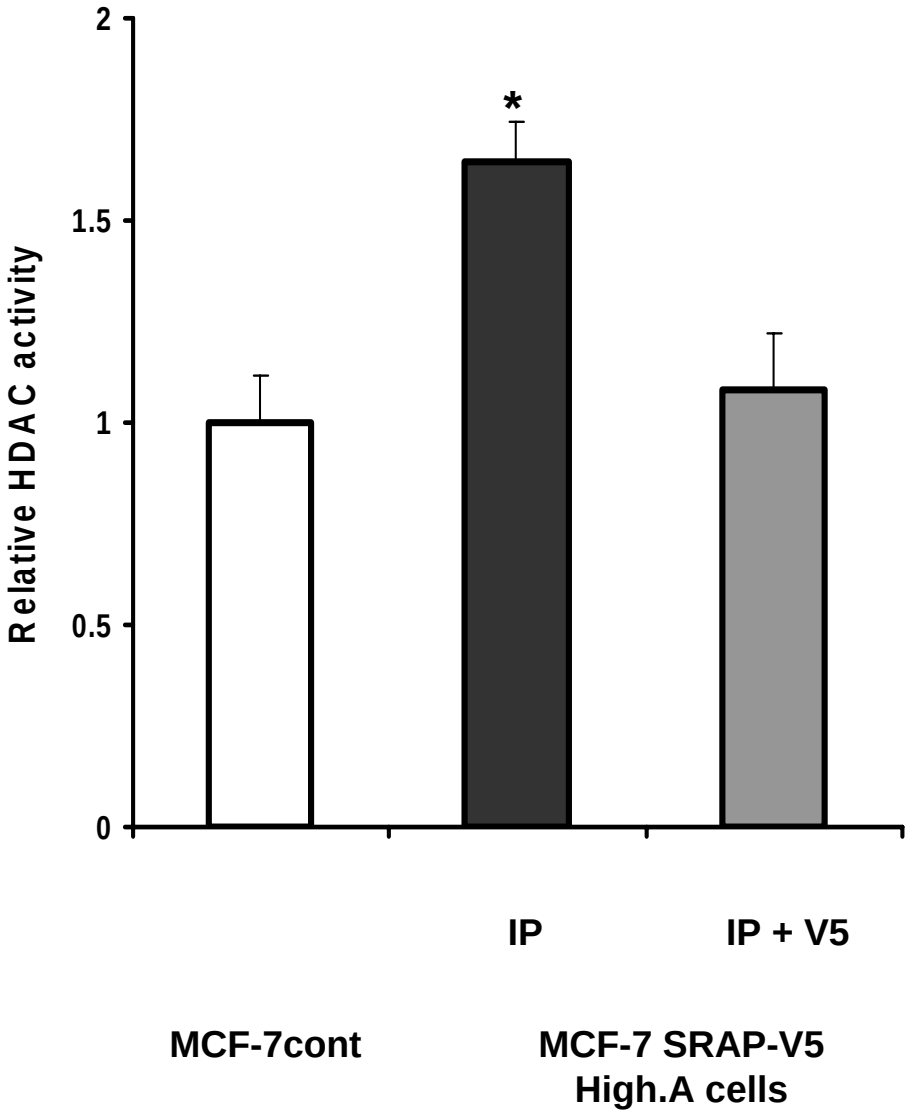
2
1.5
1
0.5
0
Relative HDAC activity
*
IP
IP + V5
MCF-7cont
MCF-7 SRAP-V5
High.A cells

Fig 7

Appendix4:

Increasing Steroid Receptor RNA Activator (SRA) intron-1 retention in human breast cancer cells using modified oligonucleotides.

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Running Title: Increasing SRA intron-1 retention

Key words: Steroid receptor RNA activator, breast cancer, oligonucleotide, alternative splicing, SRA

Abstract

The steroid Receptor RNA Activator (SRA) has originally been identified as a non-coding RNA able to co-activate steroid receptors. The existence in multiple tissues, including breast, of SRA RNAs able to encode a protein called SRAP has now however been demonstrated. This protein, as opposed to its RNA counterpart, is acting as transcriptional repressor of steroid receptor activity. The balance between these two genetically linked entities is thought to be regulated by alternative splicing of intron-1, which presence alters the reading frame and allow the co-expression, in breast cancer cells, of both non-coding and coding SRA RNAs. Herein, we have investigated the possibility to alter this balance toward the production of non-coding SRA RNA by transfection of an antisense modified oligonucleotide which reprograms SRA splicing events toward the production of more intron-1 retained SRA RNA. We first established the proof of principle of such an approach using a minigene strategy and demonstrated that oligonucleotide treatment led to the production of more intron-1 alternatively spliced RNA in T5 breast cancer cells. The resulting decrease in coding SRA, assessed by real-time PCR, was paralleled by a decrease in SRAP expression. Interestingly, the reprogramming of SRA splicing led to an increase in the expression of urokinase plasminogen activator and estrogen receptor beta genes. The critical role of these two genes in invasion processes and response to hormone, respectively, leads us to hypothesize that controlling the generation of non-coding and coding SRA RNAs might provide a new window of opportunity to fight breast cancer.

Introduction

The Steroid Receptor RNA Activator (SRA) was first identified in 1999 as a non coding RNA acting as a specific co-activator of steroid receptors (Lanz et al., 1999). In this original report, several untranslatable isoforms sharing a central core region but different in their 5' and 3' extremities were identified. Through mutation analysis, the authors unequivocally showed that the common core region, corresponding to sequences encompassing from exon-2 to exon-5 (Fig.1), was necessary and sufficient for these RNAs to act as co-activators (Lanz et al., 1999). Several studies have now been published shedding light on SRA mechanisms of action. It is now believed that SRA acts embedded in ribonucleo-protein complexes possibly recruited at the promoter of regulated genes. These complexes may contain positive regulators, such as the steroid receptor co-activator 1 (SRC-1, (Lanz et al., 1999; Watanabe et al., 2001; Hatchell et al., 2006)), DExD/H box family of RNA-helicase members p68 and p72 (Watanabe et al., 2001; Caretti et al., 2006), or pseudouridine synthases Pus1p and Pus3p (Zhao et al., 2004; Zhao et al., 2006). Negative regulators, such as SMRT/HDAC1 Associated Repressor Protein (Sharp, (Shi et al., 2001; Hatchell et al., 2006)) and the recently identified SRA stem-loop interacting RNA binding protein (SLIRP, (Hatchell et al., 2006)) also form complexes with SRA and modulate its activity. Specific secondary structural motifs of the core (Lanz et al., 2002), which participate to the formation of these complexes (Hatchell et al., 2006), have now been identified. Pseudouridylation, a common noncoding RNAs post-transcriptional modification consisting in the isomerization of specific uridine residues (U) to pseudouridine (Ψ) (Charette & Gray, 2000; Ferre-D'Amare, 2003), was also found to regulate SRA co-activator properties (Zhao et al., 2004; Zhao et al., 2006).

If SRA was originally thought to specifically co-activate steroid receptors, further data have now shown that this RNA is also co-activating non-steroid nuclear receptors including the Retinoid-Acid-Receptor (RAR, (Zhao et al., 2004; Zhao et al., 2006)) and the thyroid-receptor (TR-α1–β1, (Xu & Koenig, 2004, 2005; Hatchell et al., 2006)), as well as other transcription factors such as MyoD, a transcription factor participating to

skeletal myogenesis (Caretti et al., 2006). Altogether, SRA has a wider than first anticipated role and is likely to participate to several signalling pathways still to be uncovered.

Several coding-SRA isoforms have now been identified, which are either similar to the original SRA sequence from exon-2 to exon-5 (SRA1), or contain point mutations in exon-3 (SRA2) or exon-3 (SRA3) (see Fig.1 and (Emberley et al., 2003)). All these isoforms contain an exon-1 extended in its 5'-extremity and containing initiating methionine codons (Fig.1). It is important to note that these coding-RNAs, containing a full core (from exon-2 to exon-5), are therefore theoretically also able to act as co-activators. These coding SRAs have now been shown to lead to the production of an endogenous SRA protein (SRAP) in several tissues including breast, prostate and muscle (Emberley et al., 2003; Chooniedass-Kothari et al., 2004; Chooniedass-Kothari et al., 2006; Kurisu et al., 2006). Even though the exact functions of SRAP remain to be elucidated, it has been proposed that this protein might also regulate steroid receptor signalling, either as co-activator (Kawashima et al., 2003; Kurisu et al., 2006), or as a repressor (Chooniedass-Kothari et al., 2006; Chooniedass-Kothari S., 2006b).

We have recently identified intron-1 retention as a possible mechanism participating to the generation of coding and non coding SRA RNAs (Hube et al., 2006). We have indeed shown that fully spliced SRA transcripts co-existed in breast cancer cells with transcripts containing a full (FI) or partial (resulting from the use of an alternative 3' acceptor site located 60 bp upstream of exon-2; PI) intron-1 sequence (Fig.1, non-coding). Transcripts corresponding to an alternative 5' donor site (located 15bp upstream of the end of exon-1) and the alternative 3' acceptor site have also been identified (AD). These alternative splicing events, which either shift the SRAP open reading frame or introduce premature stop codons, make these isoforms unable to encode for SRAP but do not affect their transcriptional co-activator function as the core sequence remains intact.

SRA1 gene provides a very intriguing example of a gene that encodes products functional both at the RNA and protein levels. How such a bi-faceted and intrinsically-linked genetic system is regulated remains largely unexplored. Similarly, no studies have yet

investigated how the balance between coding and non-coding SRA RNA could be altered. In the present study, we aimed to shift the relative proportion of SRA transcripts towards more non-coding SRAs via promoting an increase in the level of intron-1 retention using a modified oligonucleotide strategy.

Results

Altering intron-1 splicing events in artificial SRA minigene transcripts using modified anti-sense oligonucleotides.

Reprogramming splicing events through transfection of modified antisense oligoribonucleosides targeting exon-intron junction has been successfully achieved in different systems (Mercatante et al., 2001a; Mercatante et al., 2001b; Mercatante & Kole, 2002). To establish the proof of principle that such an approach might be suitable to alter SRA intron-1 splicing events, we used a previously described artificial mini-gene (Hube et al., 2006) consisting of a portion of a modified *β-globin* gene, encompassing two exons flanking a constitutively splice-able intron (Dominski & Kole, 1991; Xie et al., 2005), fused to *SRA1* exon-1-intron-1-exon-2 sequence (Fig.2). Two different 2'-O-methyl-modified anti-sense oligoribonucleoside phosphorothioate 20-mers were designed to recognize the splice donor sites of β-globin (βgl-AS) and SRA introns (SRA-AS). In principle, βgl-AS and SRA-AS were expected to increase the proportion of mini-gene transcripts with β-globin intron retention and SRA intron-1 retention, respectively.

SRA-AS and βgl-AS oligos were first tested for their ability to enter cells. Cells chosen consisted in T5 breast cancer cells, previously shown to express low level of endogenous SRA RNAs retaining intron-1 (Hube et al., 2006). Cells were treated as described in the Materials and Methods section with increasing concentrations of SRA-AS and βgl-AS conjugated with the fluorophore Indocarbocyanine (SRA-AS-Cy3) and carboxyfluorescein (βgl-AS-FAM), respectively (Fig.3A). Twenty-four hours post-transfection, direct fluorescent microscopy on formaldehyde fixed cells indicated that most of the observed fluorescent signals were concentrated in nuclei as indicated by co-labelling with 4', 6-diamidino-2-phenylindole (DAPI). Cytoplasmic localization and distinct fluorescent granules at the periphery of nuclei were also observed. At concentrations lower than 0.5μM, sub-optimal transfection efficiencies were achieved. Nevertheless, 100% transfection efficiency was invariably observed when either 0.5μM of SRA-AS-Cy3 or βgl-AS-FAM oligo was used.

To determine to what extent the fluorophore-conjugated oligos persisted in T5 cells, time course experiments were performed as described in Materials and Methods. T5 cells were treated with 0.5μM SRA-AS-Cy3 or βgl-AS-FAM oligos, and fluorescent micrographs were taken at t: 24 h, 48 h , and 72h (Fig.3B). As expected, at t: 24h, T5 cells had robust fluorescent nuclear signals, corresponding to SRA-AS-Cy3 or βgl-AS-FAM; however, the proportion of cells and overall intensity of signal within individual cells decreased over time. Together these results suggest that SRA-AS-Cy3 and βgl-AS-FAM are similarly entering and retained into T5 cells.

To test whether the chosen oligos could indeed modify the splicing of transcripts produced from the *SRA-β-globin* mini-gene, T5 breast cancer cells were co-transfected with a fixed amount of mini-gene and increasing concentrations of either SRA-AS or βgl-AS oligos as described in Material and Methods. Total RNA was extracted at t: 24h and following reverse-transcription, the relative proportion of each mini-gene transcripts estimated by PCR co-amplification using upper and lower primers recognizing the first exon of β–globin and the end of SRA exon-2 mini-gene, respectively. A dose-dependent increase in the proportion of mini-gene transcripts retaining SRA intron-1 was observed upon SRA-AS treatment (Fig.4A). At the highest concentration of SRA-AS used (0.5μM), a ~6-fold increase in the relative proportion of SRA intron-1 retention was achieved (Fig.4A-B). Treatment of cells with βgl-AS similarly increased the level of β-globin intron retention in transcripts originating from the minigene (Fig.4A-C). These data provided proof of principle that SRA-AS oligo has indeed the ability to promote SRA intron-1 retention in the artificial mini-gene transcripts.

The proportion of endogenous SRA transcripts retaining intron-1 is increased following treatment of T5 cells with SRA-AS.

To further determine if the introduction of SRA-AS could also alter the balance of endogenous coding and non-coding SRA RNAs, a similar dose-response experiment was performed and the proportion of SRA intron-1 retention assessed by triple-primer PCR (TP-PCR) as described in the Materials and Methods section (Fig.5). The TP-PCR assay used to assess the balance between fully spliced and intron-1 retaining SRA transcripts

(Fig.5A) has successfully been used and validated to assess the relative proportion of transcripts sharing a common extremity but differing in the other (Leygue et al., 1996; Leygue et al., 1999a; Hube et al., 2006). This approach allows the co-amplification of two single bands, migrating at an apparent size of 377 bp and 360 bp, corresponding to non-coding (FI, PI and AD, Fig.2) and coding (fully spliced, FS) SRA (Fig.5A-B).

A dose-dependent shift towards more intron-1 retention in T5 cells when treated with SRA-AS was observed (Fig.5B). At the highest concentration tested of 0.5μM, which corresponded to a 100% transfection efficiency (Fig.3), SRA-AS led to an approximate 3 folds increase in the relative amount of intron-1 retention (Fig.5B, Lane 4), as compared to mock transfected control (Fig.5B, Lane 1). As expected, no effect was observed on the proportion of intron-1 retaining endogenous SRA RNAs when transfecting cells with similar amount of βgl oligos (Fig.5B, Lane 5-7). βgl oligonucleotides have therefore been used as negative controls in the subsequent experiments.

Lasting increase in the proportion of intron-1 retaining SRA transcripts in T5 cells treated with of 0.5μM SRA-AS.

To establish how long the increase in intron-1 retention upon treatment with SRA-AS lasted, time course experiments were performed as described in Materials and Methods. T5 cells were treated with no oligos (Mock) or with 0.5μM SRA-AS or βgl-AS oligos, and the relative expression of alternatively spliced SRA RNAs assessed by TP-PCR at t: 24 h, 48 h, and 72h (Fig.6). As expected, a significant increase (2.5 fold, $p<0.05$, Student's t-test) in relative intron-1 retention was observed at t: 24h upon SRA-AS treatment (Fig.6, line 4). A maximal 3.5-fold average increase in the relative level of intron-1 retention was observed at t: 48 hours (Fig.6, Lane 5) over that observed for time-matched mock transfected cells (Fig.6, Lane 2). This effect is maintained, albeit decreased at t: 72 hours when levels of intron-1 retention have returned to that of samples at t: 24h. (Fig.6, compare lanes 4 and 6). As expected, βgl-AS oligos transfection had no effect in promoting endogenous SRA intron-1 retention in T5 cells (Fig.6, lanes 7-9).

Increased relative expression of non-coding SRA transcripts corresponds to both an increase in intron-1 containing SRA RNAs and a decrease in the levels of fully spliced SRA RNA.

To clarify whether the relative increase in non-coding SRA RNAs expression observed by TP-PCR resulted from an increase in intron-1 retaining RNA, a decrease in fully spliced RNA or both, we assessed the expression of these SRA RNAs by real-time PCR as described in the Materials and Methods section. T5 cells were treated with no oligos (Mock), SRA-AS or βgl-AS oligos and following amplification with a lower primer annealing in exon-3 and upper primers annealing with intron 1 or exon-1, percentage modifications in the level of intron-1 retained or fully spliced quantified at: 24h. Four experiments were performed and the average modifications calculated (Fig.7). A significant ($p<0.05$, Student's t-test) average increase of 90% (corresponding to a ΔCT of 0.92) in the expression of SRA intron-1 retaining was observed in cells treated with SRA-AS as compared to mock transfection. Inversely, a significant decrease of 70% (corresponding to a ΔCT of -1.82) in fully spliced SRA RNA expression was observed. As expected, no effect of βgl-AS oligos on either intron-1 retaining or fully spliced SRA levels was observed.

Fluorophore conjugation does not alter SRA-AS effect.

Since oligos used to monitor transfection efficiency and those used to shift the balance coding/non-coding SRA RNAs towards increased non-coding SRA isoforms are not identical *per se* (i.e.one set is conjugated to a fluorophore), we were also interested to determine whether SRA-AS-Cy3 would have a similarly effect on SRA intron-1 retention in T5. T5 cells were treated with either no oligos, 0.5μM SRA-AS-Cy3 or 0.5μM βgl-AS-FAM oligos, total RNA was extracted, reverse transcribed and analyzed by TP-PCR at t: 24 h as described in Materials and Methods. An overall 2.5-fold increase in endogenous intron-1 retaining SRA RNAs was observed when transfecting with SRA-AS-Cy3 but not βgl-AS-FAM oligos (Fig.8). This 2.5-fold increase corresponded to the one observed when non-fluorescent SRA-AS oligo were used (Fig.6). Altogether these data suggest that fluorophore conjugation does not affect the splice switching function of the oligos used in this study.

Decrease of endogenous SRAP in T5 and MCF7-S151 cells treated with 0.5μM SRA-AS.

To test whether modifying the balance between coding and non-coding SRA isoforms affected the steady state levels of SRAP, we first transfected T5 cells with either SRA-AS or βgl-AS and analyzed total protein cell lysates by Western blot using anti-SRAP antibodies as described (Fig.9A). Results indicate a modest decrease in endogenous SRAP levels at t: 24 hours post-treatment with SRA-AS. This effect however becomes much more pronounced at t: 48h and 72h. No change in SRAP levels was observed in βgl-AS transfected samples.

A similar experiment was performed on MCF7-S151 breast cancer cells, which have previously been stably transfected with a cDNA construction expressing SRAP-V5 tagged protein (Emberley et al., 2003; Chooniedass-Kothari et al., 2006). Western blot analysis shows that these cells express both endogenous and transfected protein (Fig.9B). A ~50% reduction of the expression of endogenous SRAP, relative to exogenous (SRAP-V5), is observed in cells treated with SRA-AS but not in Mock or βgl-AS transfected cells (Fig.9.B).

Modulation of the balance coding/non-coding endogenous SRA RNAs alters gene expression in T5 breast cancer cells.

To establish whether the alteration of the balance coding/non coding SRA1 RNAs impacts on gene expression, total RNA from T5 cells transfected with 0.5μM SRA-AS or control βgl-AS oligos was analyzed at t: 24h by real-time quantitative PCR using a Breast Cancer and Estrogen Receptor Signaling RT^2 Profiler™ PCR Array. Four independent experiments were performed as described in the Materials and Methods section. The expression of 56 genes was evaluated in cells treated with SRA-AS and βgl-AS oligos and differences in expression assessed using the Student's t-test (see Table 2 for a full description of the genes assessed and the results obtained). Expression of five genes was significantly increased (Fig.10). A strong increase in the expression of the urokinase plasminogen activator PLAU (ΔCt = 2.87, corresponding to a 729% expression compared to the control), gene intimately linked to invasion mechanisms (Han et al., 2005) as well

as estrogen receptor beta ($\Delta Ct = 1.73$, corresponding to a 331% expression compared to the control) was observed. Similarly, expressions of Stanniocalcin 2 (STC2), the vascular endothelial growth factor (VEGF) as well as Thrombospondin 1 (THBS1) were significantly increased, even though to a lesser extend.

Discussion:

Herein we have demonstrated that the balance between fully spliced and intron-1 alternatively spliced SRA RNAs can be artificially tipped in breast cancer cells toward the production of more non-coding SRA RNA through introduction of modified oligonucleotides, complementary to the junction exon-1/intron-1.

The approach consisting in masking donor or acceptor splicing sites with modified oligonucleotides, has successfully been used by many different groups to redirect splicing events involving specific target RNAs (Karras et al., 2000; Kalbfuss et al., 2001; Mann et al., 2001; Mercatante et al., 2001a; McClorey et al., 2006; Vickers et al., 2006; Harding et al., 2007; Khoo et al., 2007). Such an approach presents several advantages. As an anti-sense method, it benefits from the fact that 2'-O-modified (either methyl or methoxy-ethyl) oligoribonucleotide phosphorothioates bind to pre-mRNA target sequences *in vivo* with high specificity (Stein, 1997) but do not form RNase H competent RNA-oligonucleotide hybrid complexes. Therefore, unlike more classical anti-sense oligonucleotides techniques that result in mRNA degradation, these modified anti-sense oligos do not affect RNA stability but rather serve as negative regulators through masking splice recognition sequences thereby preventing recruitment of splicing factors. Applied to the endogenous SRA RNA population, which ultimately consists in fully spliced coding and intron-1 alternatively spliced non coding species, this strategy allows to reprogram the fate of immature RNAs toward the production of more non coding RNAs. Reprogramming endogenous RNAs, as opposed to introducing exogenous non-coding RNAs under the control of artificial promoter such as the Cytomegalovirus promoter (CMV), presents an obvious advantage to interrogate physiological balance modifications. Treatment of cells with SRA-AS oligonucleotides allowed the relative intron-1 retention levels to increase by a factor of 2.5 folds. This increase is very similar to the difference previously observed (2 folds) between MDA-MB-468 invasive breast cancer cells and T5 non invasive breast cancer cells (Hube et al., 2006). This confirms the suitability of this approach to alter the balance coding/non coding SRA RNAs in physiological proportion. Altogether, the approach used appears adequate for

reprogramming the balance coding/non coding SRA RNAs toward different physiological levels observed in the various cell lines previously characterized.

We have observed that fluorescent oligonucleotides had a similar effect on altering SRA splicing as compared to non-fluorescent molecules. The possibility to label such oligonucleotides with fluorescent tag allows the quick assessment of transfection efficiency and individual identification of transfected cells. This later property could also allow subsequent co-labelling experiments aiming at assessing whether transfected cells are indeed the ones expressing more or less of a given genes identified by other means (such as real-time PCR or Western blot) as differentially expressed under oligonucleotide treatment.

We showed that the increase (2.5 folds) in the relative amount of intron-1 retained SRA RNA as assessed by TP-PCR corresponded, using real-time PCR, to an absolute increase of ~90% of this transcript and a ~70% decrease of fully spliced SRA RNA (Fig.7). As expected, the decrease in coding SRA RNA resulted in a decrease in endogenous SRAP levels detected by Western blot (Fig.9A). To further establish whether the action of SRA-AS on SRAP level was indeed mediated through modulation of splicing events and not through other mechanisms such as translation inhibition we investigated the effect of this oligonucleotide on cells expressing both endogenous and exogenous fully spliced SRA RNA. MCF7-S151 cells have indeed previously been engineered to stably express a V5-epitope tagged SRAP (SRAP-V5) from an expression vector corresponding to fully spliced SRA cDNA (Emberley et al., 2003; Chooniedass-Kothari et al., 2004). As such, two populations of SRAP-coding mRNAs exist within these cells: an exogenous one, fully spliced, of plasmid origin coding for SRAP-V5; and an endogenous one, which has to be fully spliced by the splicing machinery to produce the endogenous SRAP. Since the oligos were designed such as only 3 nucleotides fall within intron-1 (the remaining 17 nucleotides fully aligns with 3' extremity of exon-1), the hybridization efficiency of SRA-AS to exogenous V5-SRAP mRNA or endogenous coding fully-spliced SRAP mRNA is likely to be the same. Therefore if SRA-AS acts as translation inhibitor or as frame-shift inducer, we would expect a similar decrease in V5-SRAP levels upon SRA-AS treatment. Only a decrease in endogenous SRAP levels occur 48-72h post-treatment with 0.5μM SRA-AS (Fig.9), while exogenous V5-SRAP levels remains unaffected. This data

supports the notion that indeed the decrease of SRAP results from a splice switching action of SRA-AS oligonucleotide.

Among the genes whose expression was modified upon SRA-AS oligonucleotide treatment figured PLAU, the urokinase plasminogen Activator also called uPA, known, through the activation of several metalloproteinase, to play a critical role in the development of metastases (Duffy, 2004; Han et al., 2005). It is interesting to note that we have previously reported that invasive MDA-MB-231 and MDA-MB-468 breast cancer cells expressed significant more SRA RNAs retaining intron-1 than non invasive MCF-7, T47D or T47D cells, whereas the more "normal" MCF-10A1 breast cell line expressed the lowest relative level of SRA intron-1 RNA (Hube et al., 2006). This suggested that a balance "tipped" toward the production of non-coding SRA1 RNA in breast cells might affect growth and/or invasion properties. Altogether, these observations elude to the fact that non-coding SRA RNA, through the over-expression of genes such as PLAU, might therefore directly participate to the establishment of an invasive phenotype in breast cancer cells. Further studies are needed to corroborate this hypothesis.

We observed a strong increase in ESR2 expression upon SRA-AS treatment. ESR2, also known as estrogen receptor beta, is believed through modulation of the response to endogenous estrogens, to participate in the mechanisms underlying breast tumorigenesis and tumor progression (Murphy et al., 2003; Imamov et al., 2005; Saji et al., 2005; Murphy & Watson, 2006). ESR2 expression has been previously shown to be up-regulated by estrogens in breast cancer cells expressing estrogen receptor alpha (Cappelletti et al., 2003). Similarly, the expression of three other genes, Stanniocalcin 2, Vascular endothelial growth factor and Thrombospondin 1, known to be up-regulated by estrogens in MCF-7 breast cancer cells (Ghosh et al., 2000; Frasor et al., 2003), was also increased in our experiments when T5 cells were treated by SRA-AS. Non coding SRA RNA is a co-activator of estrogen receptor alpha (Lanz et al., 1999; Cavarretta et al., 2002; Deblois & Giguere, 2003) and T5 cells contain this receptor. It is therefore not surprising that the expression of such target genes is modified upon treatment with SRA-AS, which leads to the production of more non-coding SRA RNAs. However, a possible effect of the concurrently observed decrease in SRAP protein level (observed by Western

blot) cannot be excluded. Indeed, we have recently proposed that SRAP itself might participate in regulating estrogen receptor signaling in breast cancer cells (Chooniedass-Kothari et al., 2006; Chooniedass-Kothari S., 2006b, a). Further studies are needed to establish the respective participation of SRA RNA and SRAP in the mechanisms leading to the over-expression of the genes mentioned above.

The full sequencing of the human genome has led to a renewed awareness of alternative splicing events. Indeed, originally thought to possibly result from “hiccups” of the splicing machinery, these events have now been shown to be highly controlled and to introduce a new layer of complexity in the regulation of gene expression (Brett et al., 2002; Modrek & Lee, 2002; Roberts & Smith, 2002; Lemischka & Pritsker, 2006). Strategies, aiming at favoring the production of a given splice variant are currently developed and proposed as new therapeutic tools.(Puttaraju et al., 1999; Mercatante et al., 2001a; Mercatante et al., 2001b; Mercatante & Kole, 2002; Mansfield et al., 2003; Mansfield et al., 2004; Garcia-Blanco, 2005; Hagiwara, 2005; Wilton & Fletcher, 2005; Yang & Walsh, 2005; Hayes et al., 2006).

Herein, we have shown that the balance coding/non-coding SRA RNAs could be altered through the use of modified oligonucleotides in breast cancer cells. This led to a change in expression of genes such as PLAU and ESR2, likely to have an important impact on two critical aspects of breast cancer cell phenotypes, namely invasion and response to estrogen. Additional studies are needed to characterize the phenotype modifications of cell upon oligonucleotide treatment and establish whether modifying SRA splicing events might lead to establishing new breast cancer treatments.

Material and Methods

Cell culture

T5 breast cancer cell line was kindly provided by Dr. LC. Murphy (Coutts et al., 1996) and MCF-7 stably transfected with pCDNA 3.1 SRAP-V5 His (MCF-7-S151) were previously described (Emberley et al., 2003). All cells were cultured in DMEM (GIBCO, Grand Island, NY) medium supplemented with 5% fetal bovine serum (CANSERA, Rexdale, ON), penicillin (100 units/ml), streptomycin (100 μg/ml) (GIBCO, Grand Island, NY), and 0.3% glucose. Cells were grown in a 37°C humidified incubator with 5% CO_2.

Oligonucleotide Treatment

2'-O-Methyl-oligoribonucleoside phosphorothioate 20-mers anti-sense to the 5'-splice site of SRA intron-1 (SRA-AS, ACCCGGCUUCACGUACAGCU) and to the 5'-splice site of a modified β–globin intron (βgl-AS, ACCUGCCCAGGGCCUCACCA), as well as fluorophore conjugated versions of the aforementioned oligoribonucleotides were synthesized and purified by Trilink Biotechnologies, Inc. (San Diego, CA). Oligonucleotides were transfected into cells with DMRIE-C reagent (16 μg/ml; Invitrogen) according to the manufacturer's directions at the concentrations indicated in the figures. Five hours post-treatment, transfection medium was replaced with fresh medium, and cells were cultured for the indicated times.

For oligonucleotide and minigene co-transfection...see Jimin

Fluorescent Microscopy

Cells were cultured on cover-slips and transfected with fluorophore-conjugated oligonucleotides at the indicated concentrations and times post-treatment. Coverslips were then briefly washed with PBS and cells were fixed with 3.7% formaldehyde in PBS for 15 minutes at room temperature. Cover-slips were then rinsed with PBS and cells permeabilized with 0.2% Triton-X100 in PBS for 1-2 minutes prior to staining nuclei with 1μg/ml *4',6-diamidino-2-phenylindole-dihydrochloride* (Dapi). Coverslips were mounted with FluorSave™Reagent (Calbiochem, La Jolla, CA). Fluorescent images were

captured with an Eclipse E1000 epifluorescent microscope (Nikon), digitized with ACT-1 software (v.2.63; Nikon) and merged images were generated with Photoshop 6.0 (Adobe)

RT-PCR, Triple-primer –PCR (TP-PCR).

Total RNA was extracted from cells using Trizol™ reagent (Gibco BRL, Grand Island, NY) according to the manufacturer's instructions. Half a µg of total cellular RNA was reverse transcribed in a final volume of 30 µl using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase and random hexamers as previously described (Emberley et al., 2003). One an a half µl of reverse-transcription mixture was amplified in a final volume of 15 µl, in the presence of 60 mM Tris-HCl (pH 8.5), 15 mM $[NH_4]^2SO_4$, 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 4 ng/µl of each primer (Pairs or three primers for RT-PCR and TP-PCR, respectively), 1 unit of Platinum Taq DNA polymerase (Invitrogen Carlsbad, CA) and 10nM α-^{32}P dCTP. Each PCR consisted of a 5 minutes pre-incubation step at 94°C followed by 30 cycles of amplification (30 sec at 94°C, 30 seconds at 60 °C, and 30 seconds at 72 °C). The sequences of primers used are detailed in Table 1.

Radio-labeled PCR products were then separated on poly-acrylamide gels as previously described (Leygue et al., 1999b). Following electrophoresis, the gels were dried and exposed 30 minutes to a Molecular Imager™-FX Imaging screen (Bio-Rad, Hercules, CA). Exposed screen was then scanned using a Molecular Imager™-FX (Bio-Rad, Hercules, CA), which allows the subsequent quantification of each observed signal.

Quantification of PCR signals

PCR signals were quantified using a Molecular Imager™-FX (Bio-Rad, Hercules, CA) as previously described (Leygue et al., 1999b). For assessing the relative amount of minigene transcripts (Figure 4), the signal corresponding to the transcript of interest (FI or βgl-intron-signal) was expressed for each treatment as a percentage of the total signal measured (FI+FS+PI+βgl-intron). To obtain a value for intron retention in arbitrary units (Intron-1 a.u or βgl-intron a.u, Figure 4), the calculated percentage was divided by the percentage observed with no treatment (corresponding to 1 a.u). For assessing the

expression of intron-1 SRA relative to fully spliced SRA in TP-PCR reactions, signals corresponding to intron-1 and fully spliced were similarly quantified. The ratio found in Mock transfected cells was used as reference (corresponding to 1 a.u, Figure 5, 8). For figure 6, four experiments were performed and for each time the average signal calculated. The average obtained in Mock transfected cells was used as reference (corresponding to 1 a.u). Bars represent standard deviation. For each time and experiment, differences with the signal obtained in mock cells were tested using the Student's t-test (two sided, paired).

Western Blot analysis.

Total cell lysates were analyzed and SRAP was detected using rabbit anti-SRAP 743 (Bethyl, Montgomery, TX) antibody in conjunction with a goat anti-rabbit HRP (Sigma, St Louis, MO) antibody at dilutions of 1/1000 and 1/3000 respectively as described (Chooniedass-Kothari et al., 2006). The level of endogenous SRAP was expressed as a percentage of the total signal corresponding to the sum of signals obtained for SRAP-V5 and SRAP (Fig.9). The value found in mock transfected cells was then used as reference (taken at 1 a.u).

Real-time PCR (Fig.7).

blabla...Need primer sequence reference used (36B4, references)...establishment of CT values....

Quantification of signals: Four experiments were performed. For each treatment and experiment, the

number of cycles needed to obtain a signal for fully spliced and intron-1 alternatively spliced was first corrected for the number of cycle leading to the average 36B4 signal to obtain a normalized cycle number. The average number of normalized cycles for intron-1 retained and Fully spliced RNA from the four experiments performed with no oligo (Mock) was then calculated to obtain Intron-1 average mock and fully spliced average mock. For each treatment and experiment, the normalized cycle number of intron-1 retained and fully spliced SRA RNA was then subtracted from Intron-1 average mock

and fully spliced average mock, respectively, to get the number of cycle difference between treatments (ΔCt) and establish the standard deviations. A ΔCt of +1 represents a 2^1 = 2 fold increase in expression, whereas a ΔCt of -2 represents a 2^2 = 4 fold decrease in expression. Differences between Mock and oligo treatments were tested using the Student's t-test (two sided).

Real-time PCR array (Fig.10).

blabla, say we checked by TP-PCR. establishment of Ct values....

Quantification of signals: Four experiments were performed. For each treatment, gene and experiment, the number of cycles needed to reach the threshold of was first corrected for the number of cycle leading to the β-actin signal (ACTB) to obtain the corrected cycle numbers. For each gene, the average number of corrected cycles from the four experiments performed with βgl-AS was then calculated, leading to the reference gene-βgl-AS. For each treatment (SRA-AS and βgl-AS) the corrected cycle number was then subtracted from the reference gene-βgl-AS to get the number of cycle difference between treatments (ΔCt) and establish the standard deviations. Differences between gene expression upon SRA-AS and βgl-AS treatments were tested using the Student's t-test (two-sided). Levels of expression presented in Table 2 were calculated as follow. If ΔCt was higher than or equal to 0, expression corresponds to 100 x $2^{\Delta Ct}$ whereas when ΔCt is lower than 0 (i.e negative), expression corresponds to $100/2^{-\Delta Ct}$.

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TABLES

Table 1: Sequence of primers used.

Primer	Sequence	Used in	
A	GTGCACCTGACTCCTGAGGAGAA	Fig.4	Amplification of mini-gene corresponding transcripts
B	CTCTGGGGGATCCATCCTGGGGTG	Fig.4	Amplification of mini-gene corresponding transcripts
C	CCCCAGTATAAGCTAACAGT	Fig.5-8	TP-PCR and real time PCR for Intron-1 retained RNA
D	GCCAAGCGGAAGTGGAGAT	Fig.5-8	TP-PCR and real time PCR for Intron-1 retained RNA
E	GACGTCTTCCAATGCCTGTT	Fig.5-8	TP-PCR and real time PCR for Intron-1 retained RNA

Table 2: Change in gene expression at t: 24h following treatment of T5 cells with SRA-AS

Symbol	Description	ΔCT	% expression	p value
PLAU	**Plasminogen activator, urokinase**	**2.87**	**729**	**0.021**
ESR2	**Estrogen receptor 2 (ER beta)**	**1.73**	**331**	**0.029**
STC2	**Stanniocalcin 2**	**1.20**	**230**	**0.009**
JUN	V-jun sarcoma virus 17 oncogene homolog (avian)	1.15	222	0.164
VEGF	**Vascular endothelial growth factor**	**0.98**	**197**	**0.034**
SERPINA3	Serpin peptidase inhibitor, clade A , member 3	0.84	179	0.187
ID2	Inhibitor of DNA binding 2	0.72	165	0.234
THBS1	**Thrombospondin 1**	**0.58**	**149**	**0.043**
NGFR	Nerve growth factor receptor	0.55	146	0.404
SLC7A5	Solute carrier family 7 , member 5	0.45	137	0.433
DLC1	Deleted in liver cancer 1	0.43	134	0.202
KLF5	Kruppel-like factor 5 (intestinal)	0.43	134	0.369
CCNA2	Cyclin A2	0.33	125	0.223
CLDN7	Claudin 7	0.25	119	0.523
HMGB1	High-mobility group box 1	0.23	117	0.637
IL6ST	Interleukin 6 signal transducer (gp130, oncostatin M receptor)	0.20	115	0.620
ITGB4	Integrin, beta 4	0.12	109	0.642
TOP2A	Topoisomerase (DNA) II alpha 170kDa	0.12	108	0.819
ERBB2	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2	0.07	105	0.898
MUC1	Mucin 1, transmembrane	0.05	104	0.834
TP53	Tumor protein p53 (Li-Fraumeni syndrome)	0.05	104	0.915
MKI67	Antigen identified by monoclonal antibody Ki-67	0.05	104	0.867
HPRT1	Hypoxanthine phosphoribosyltransferase 1	0.05	104	0.922
TFF1	Trefoil factor 1	0.03	102	0.951
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	0.00	100	1.000
ACTB	Actin, beta	-	100	
CCND1	Cyclin D1	0.00	100	1.000
TGFA	Transforming growth factor, alpha	0.00	100	1.000
RPL27	Ribosomal protein L27	-0.03	98	0.959
PTEN	Phosphatase and tensin homolog	-0.03	98	0.902
CTNNB1	Catenin (cadherin-associated protein), beta 1, 88kDa	-0.03	98	0.938
CLU	Clusterin rotein J)	-0.03	98	0.868
RPL13A	Ribosomal protein L13a	-0.03	98	0.894
CDKN1B	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	-0.05	97	0.830
ESR1	Estrogen receptor 1	-0.05	97	0.878
NME1	Non-metastatic cells 1, protein (NM23A) expressed in	-0.07	95	0.850
KRT19	Keratin 19	-0.08	95	0.725
ITGA6	Integrin, alpha 6	-0.08	94	0.869
SERPINE1	Serpin peptidase inhibitor, clade E , member 1	-0.10	93	0.857
BCL2	B-cell CLL/lymphoma 2	-0.10	93	0.797
CTSD	Cathepsin D (lysosomal aspartyl peptidase)	-0.14	91	0.740
GABRP	Gamma-aminobutyric acid (GABA) A receptor, pi	-0.15	90	0.760
CCNE1	Cyclin E1	-0.17	89	0.473
KRT18	Keratin 18	-0.18	89	0.660
GSN	Gelsolin (amyloidosis, Finnish type)	-0.20	87	0.706
CTSB	Cathepsin B	-0.22	86	0.654
SERPINB5	Serpin peptidase inhibitor, clade B (ovalbumin), member 5	-0.23	85	0.677
HSPB1	Heat shock 27kDa protein 1	-0.25	84	0.658
EGFR	Epidermal growth factor receptor	-0.26	84	0.222
GATA3	GATA binding protein 3	-0.27	83	0.305
FAS	Fas (TNF receptor superfamily, member 6)	-0.35	78	0.142
NFYB	Nuclear transcription factor Y, beta	-0.43	74	0.102
IGFBP2	Insulin-like growth factor binding protein 2, 36kDa	-0.43	74	0.357

CD44	CD44 antigen (Indian blood group)	-0.47	72	0.148
BAG1	BCL2-associated athanogene	-0.55	68	0.129
IL6R	Interleukin 6 receptor	-0.70	62	0.088

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Figure legends

Figure 1:

Coding and non-coding SRA transcripts arise from alternative splicing of SRA intron-1 in human breast cancer cells.

SRA1 gene, located on chromosome 5q31.3, consists of 5 exons (boxes) and 4 introns (plain lines). The originally described SRA sequence (AF092038) contains a core sequence (light gray), necessary and sufficient for SRA RNAs to act as co-activators (Lanz et al., 1999). Three coding isoforms have now been identified (SRA1, SRA2, SRA3), which mainly differ from AF092038 by an extended 5'-extremity containing AUG initiating codons (vertical white bar in exon 1). The stop codon of the resulting open reading frame is depicted by a black vertical bar in exon5. Black stars in exon 2 and 3 correspond to a point mutation (position 98 of the core: U to C) and a point mutation followed by a full codon (position 271 of the core: G to CGAC), respectively. Three non-coding SRA isoforms containing a differentially-spliced intron-1 have been characterized: FI, full intron-1 retention; PI, partial intron-1 retention; AD, alternative 5' donor and partial intron retention. Thick straight line, 60 bp of intron 1 retained in PI; triangulated lines represent splicing events.

Figure 2:

Splice-switching strategy used to modify *β-globin*/*SRA* mini-gene transcripts.

Schematic illustration of a portion of the previously described *β-globin*/*SRA* fusion mini-gene used in this study (Hube et al., 2006). Exons and introns are represented by boxes and plain lines, respectively. *β-globin* exons, *SRA* exon-1 and *SRA* exon-2 are in white, dark gray and light gray, respectively. βgl-AS (white bar) and SRA-AS (dark gray bar) 2'-O-methyl phosphorothioate oligoribonucleotides that target 5' donor-splice sites are depicted. βgl-AS and SRA-AS are expected to increase the proportion of mini-gene transcripts with β-globin and SRA intron-1 retention, respectively. The locations of the upper (A, white arrowhead) and lower (B, light gray arrowhead) PCR primers subsequently used to amplify mini-gene cDNAs are also indicated. Triangulated line

represents β-globin intron splicing; dashed triangulated line illustrates the differential splicing events of SRA intron-1.

Figure 3:

Uptake of fluorophore conjugated SRA-AS-cy3 and βgl-AS-FAM by T5 breast cancer cells. A) T5 cells were treated with increasing concentration (0.05, 0.1, 0.5 μM) of fluorophore-conjugated oligonucleotides SRA-AS-Cy3 (red signal) or βgl-AS-FAM (green signal). Transfection efficiency was monitored by direct fluorescent microscopy on formaldehyde fixed cells 24 hours post-treatment as described in Materials and Methods. Nuclear staining was perfomed using DAPI (4',6-diamidino-2-phenylindole-dihydrochloride).

B) T5 cells were treated with 0.5μM SRA-AS-Cy3 (red signal) or βgl-AS-FAM (green signal) and fluorescent microscopy performed at t: 24 h , 48 h and 72 h as described in Materials and Methods. White bars correspond to 10μm.

Figure 4:

Shift in splicing of SRA minigene products with SRA-AS and βgl-AS oligos.

T-5 cells were co-transfected with SRA minigene and increasing concentrations (0.05 μM, 0.1 μM and 0.5 μM) of modified anti-sense oligos (SRA-AS or βgl-AS) as described in the Materials and Methods section.

A) Total RNA was extracted at t: 24h, reverse transcribed and amplified by PCR using primers recognizing βgl exon-1 and SRA exon-2. PCR products were separated on PAGE gels and visualized as previously described (Hube et al., 2006). PCR products corresponding to transcripts with full SRA intron-1 retention (FI), βgl-intron retention (βgl-I), partial SRA intron-1 retention (PI), fully spliced (FS) or containing the alternative donor site (AD) were identified.

B) Signal corresponding to SRA full intron-1 retention transcript (FI) was quantified in cells treated with SRA-AS and expressed in arbitrary unit, as described in the Materials and Methods section. **C)** Signal corresponding to βgl intron retention transcript (βgl-I) was quantified in cells treated with βgl-AS and expressed in arbitrary unit, as described in the Materials and Methods section.

Figure 5:

SRA-AS oligos increase the relative amount of endogenous intron-1 retaining SRA transcripts.

A) Principle of TP-PCR amplification used to assess the relative proportion of coding and non-coding SRA mRNAs. Three primers are used during the PCR reaction: a lower primer (primer E) recognizing a shared region in exon-3 and two upper primers, specific for exon-1 (primer D) and intron-1 (primer C) sequences, respectively. Primer C has been chosen to recognize all intron-1 alternative splicing events (FI, PI and AD, see Fig.2), generating a single PCR product of 377 bp corresponding to non-coding SRAs (intron-1), whereas the D primer will participate to the amplification of a 360 bp fragment corresponding to fully spliced coding SRA (FS).

B) T5 cells were treated with increasing amounts (0.05, 0.1, 0.5 μM) of SRA-AS or βgl-AS oligos. At t:24 h, total RNA was extracted, reverse-transcribed and amplified by TP-PCR as described in Material and Methods. *Upper panel:* Samples were separated on PAGE gel and visualized using a Molecular Imager™-FX. *Lower panel:* Signals corresponding to intron-1 and FS were quantified and the proportion of intron-1 retention expressed in arbitrary unit (a.u) as described in Materials and Methods.

Figure 6:

Lasting effect of 0.5μM SRA-AS on the relative proportion of intron-1 retaining SRA transcripts in T5 cells.

T5 cells were treated with no oligos (Mock), 0.5 μM of SRA-AS or 0.5 μM βgl-AS oligos. At t: 24h, 48h and 72h, total RNA was extracted, reverse-transcribed and amplified by TP-PCR as described in Material and Methods.

A) Samples were separated on PAGE gel and visualized using a Molecular Imager™-FX.

B) Signals corresponding to intron-1 and FS were quantified and the proportion of intron-1 retention expressed in arbitrary unit (a.u) as described in Materials and Methods. Bars represent the average value of 3 independent experiments normalized to values obtained for mock transfection at a given time point. Error bars represent standard deviation. * corresponds to p values lower than 0.05 (Student's t-test).

Figure 7:

Quantification of intron-1 retaining and fully spliced SRA RNAs expression by real-time PCR following treatment of T5 cells.

T5 cells were treated with no oligos (Mock, white square), 0.5 μM of SRA-AS (black square) or 0.5 μM βgl-AS oligos (gray square). At t: 24h, total RNA was extracted, reverse-transcribed and analyzed by real-time PCR as described in Material and Methods. Four experiments were performed and for each treatment, ΔCt has been calculated as described in the Materials and Methods section. Dots represent the average ΔCt for each treatment whereas bars correspond to standard deviations. * indicate a significant difference (Student's t-test, two sided) in the corresponding SRA (intron-1 alternatively spliced or fully spliced) expression between mock and oligos- transfected cells.

Figure 8:

Similar effect of Fluorescent SRA-AS-cy3 and SRA-AS oligos.

T5 cells were treated with no oligo (Mock), 0.5μM SRA-AS-Cy3 or 0.5μM βgl-AS-FAMRT and the relative proportion of intron-1 retaining SRA Transcripts assessed by TP-PCR as described in the Materials and Methods section.

A) Samples were separated on PAGE gel and visualized using a Molecular Imager™-FX. Fragments corresponding to alternatively spliced SRA RNAs (intron-1) and fully spliced coding SRA (FS) are detected.

B) Signals corresponding to intron-1 and FS were quantified and the proportion of intron-1 retention expressed in arbitrary unit (a.u) as described in Materials and Methods.

Figure 9:

Western blot analysis of SRAP in T5 and MCF7-S151 cells treated with 0.5μM SRA-AS over time.

T5 and MCF7-S151 **(**engineered to stably express a V5-epitope tagged SRAP (V5-SRAP), were treated with 0.5 μM of SRA-AS or 0.5 μM βgl-AS oligos. At t: 24h, 48h and 72h, total were extracted and analyzed by Western using anti-SRAP antibodies as described in Material and Methods.

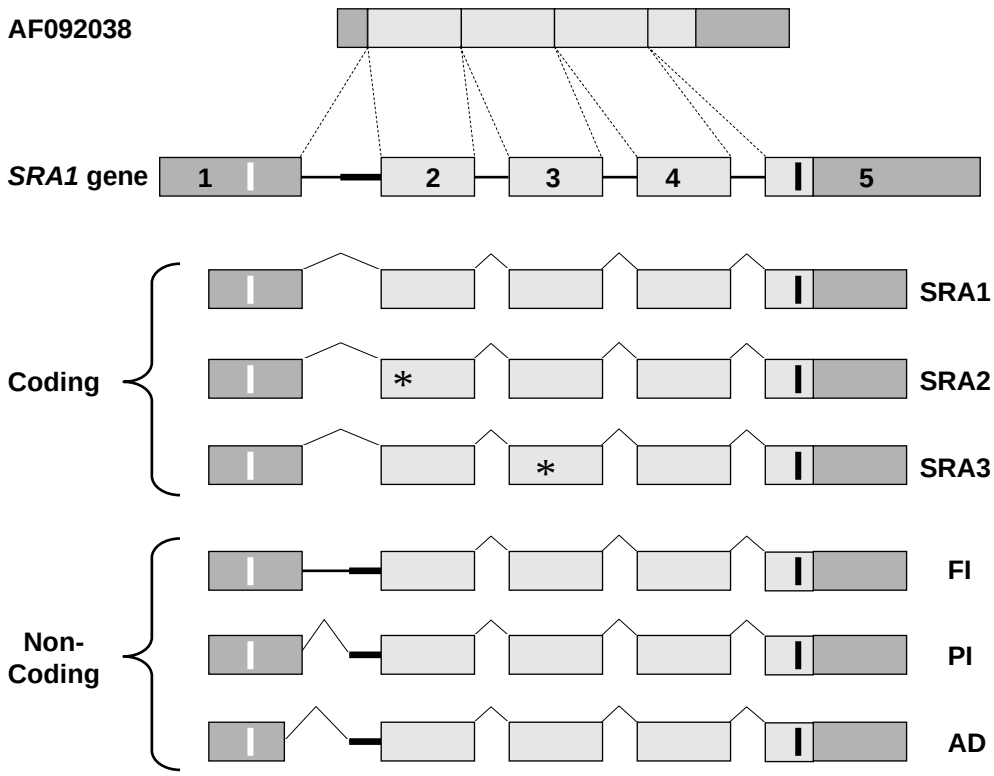
A) *Top panel*, A single band corresponding to endogenous SRAP is seen in T5 cells *Bottom panel,* A Commassie stained gel run in parallel is shown for loading control.

B) *Top panel,* Two bands, corresponding to endogenous and V5-tagged SRAP, respectively, are detected in MCF7-S151 cells. *Lower panel,* For each time and treatment, signals have been quantified and endogenous SRAP signal expressed in arbitrary unit (a.u), as described in Materials.

Figure 10:

Change in gene expression in T5 at t: 24h when treated by SRA-AS.

T5 cells were treated with 0.5 μM of SRA-AS or 0.5 μM βgl-AS oligos. At t: 24h, RNA was extracted, reverse-transcribed, checked by TP-PCR for modification of intron-1 retention and used to assess, by real-time PCR, the expression of a series of 56 genes historically linked to breast cancer, as described in the Materials and Methods section. Four independent experiments were performed. Blue dots represent, for each gene, the normalized expression upon βgl-AS oligos treatment. Orange dots represent the average ΔCT increase or decrease in gene expression upon SRA-AS treatment. Bars represent the standard deviation for each gene and treatment. Genes whose expression is significantly modified ($p<0.05$, Student's-t-test, two sided) upon SRA-AS treatment are indicated by orange boxes.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SRA1 gene
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3
4
5
Coding
SRA1
SRA2
SRA3
*
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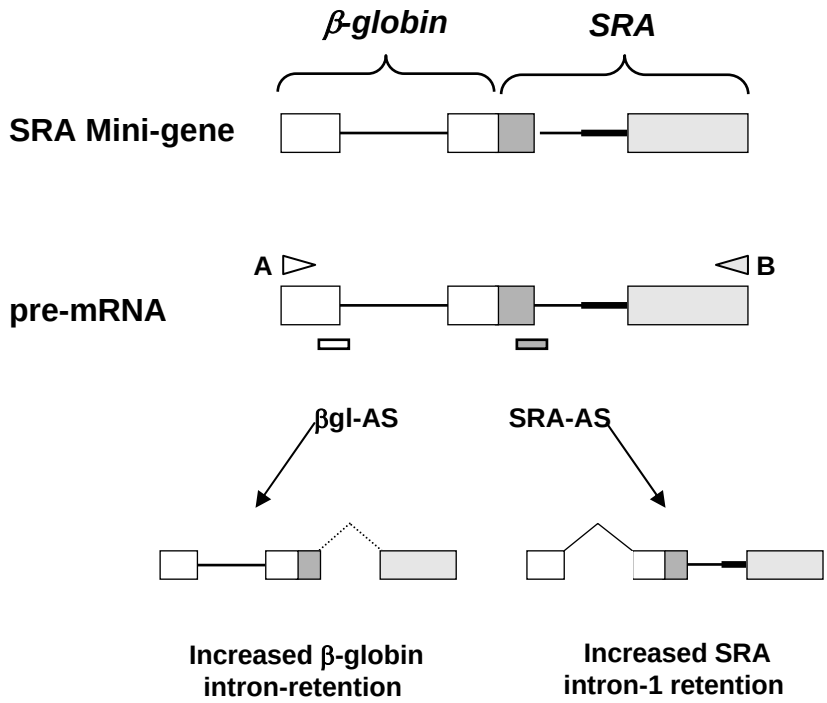
Non-
Coding
FI
PI
AD

Fig.1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SRA
SRA Mini-gene
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B
pre-mRNA
βgl-AS
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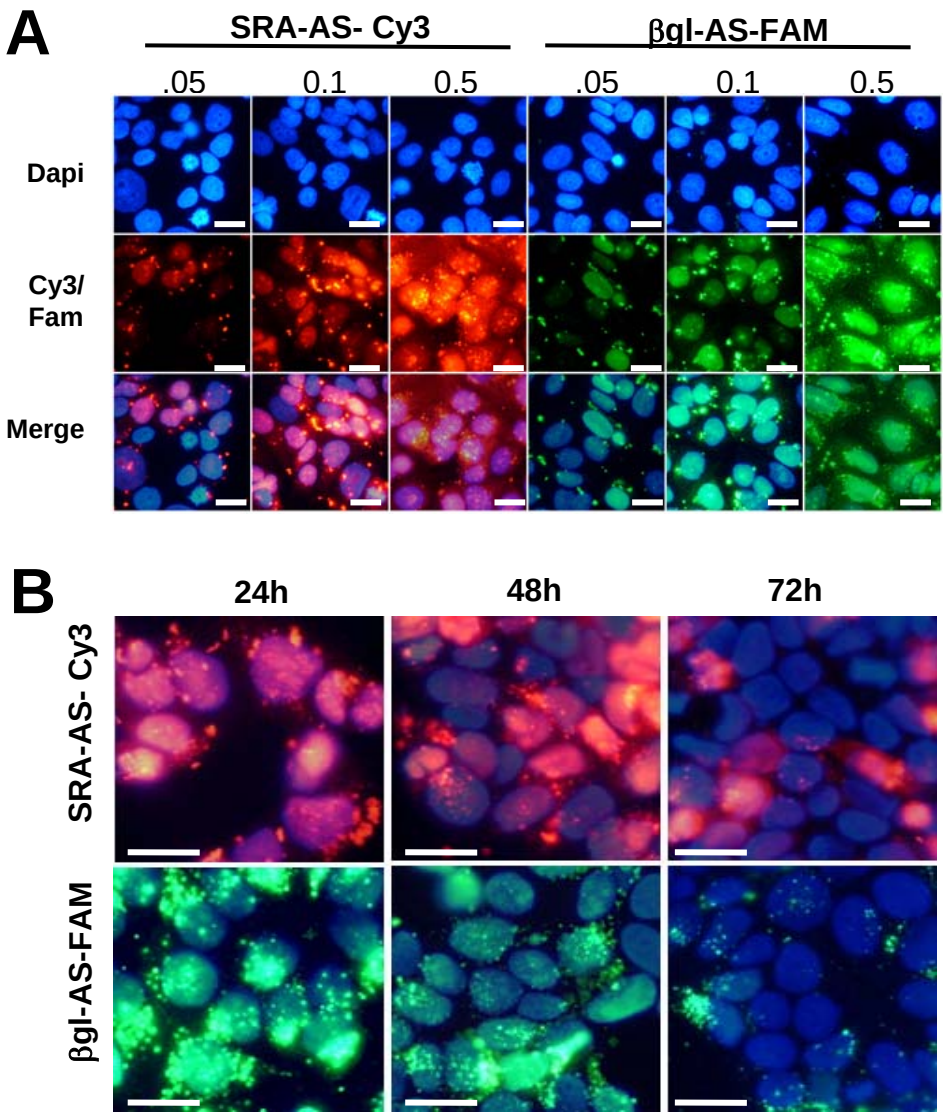
Increased SRA intron-1 retention

Fig.2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βgl-AS-FAM
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0.1
0.5
.05
0.1
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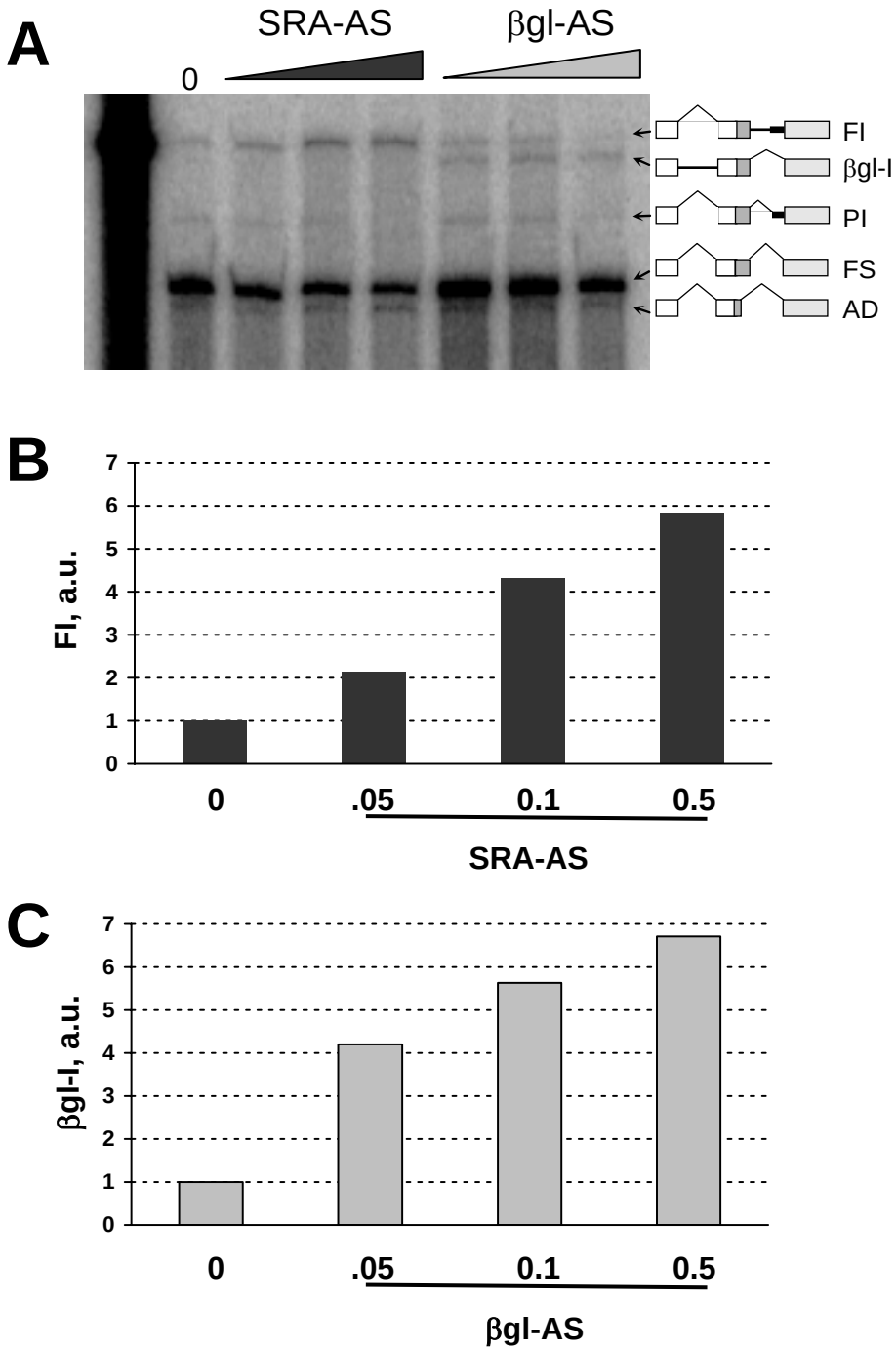
Dapi
Cy3/
Fam
Merge
B
24h
48h
72h
SRA-AS- Cy3
βgl-AS-FAM

Fig.3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AD
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5
6
7
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0.1
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SRA-AS
C
βgl-I, a.u.
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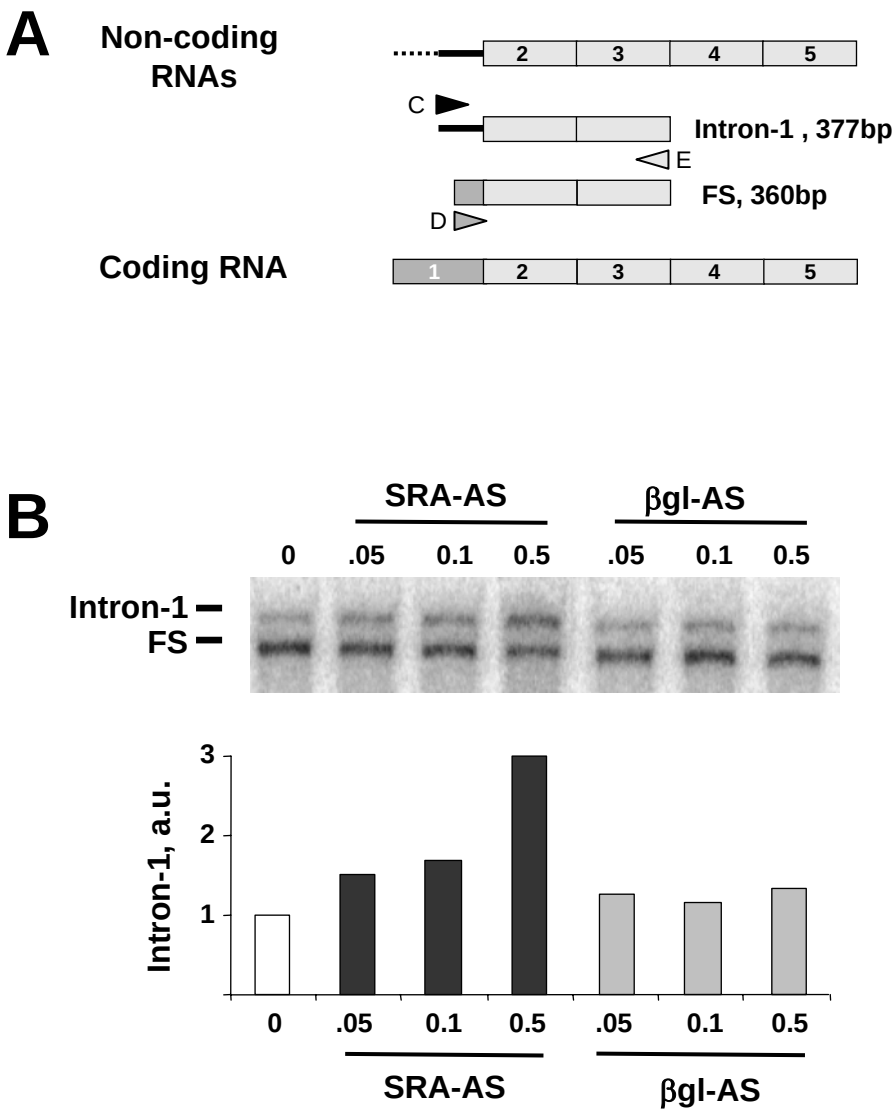
βgl-AS

Fig.4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FS, 360bp
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4
5
B
SRA-AS
βgl-AS
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.05
0.1
0.5
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Intron-1
FS
Intron-1, a.u.
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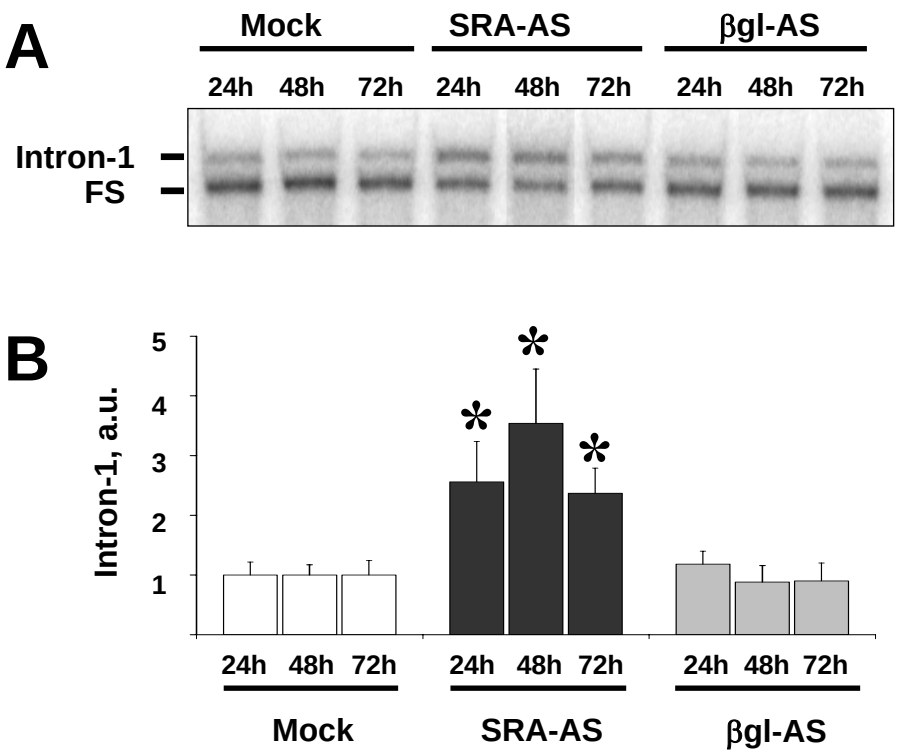
βgl-AS

Fig.5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βgl-AS
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FS
B
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4
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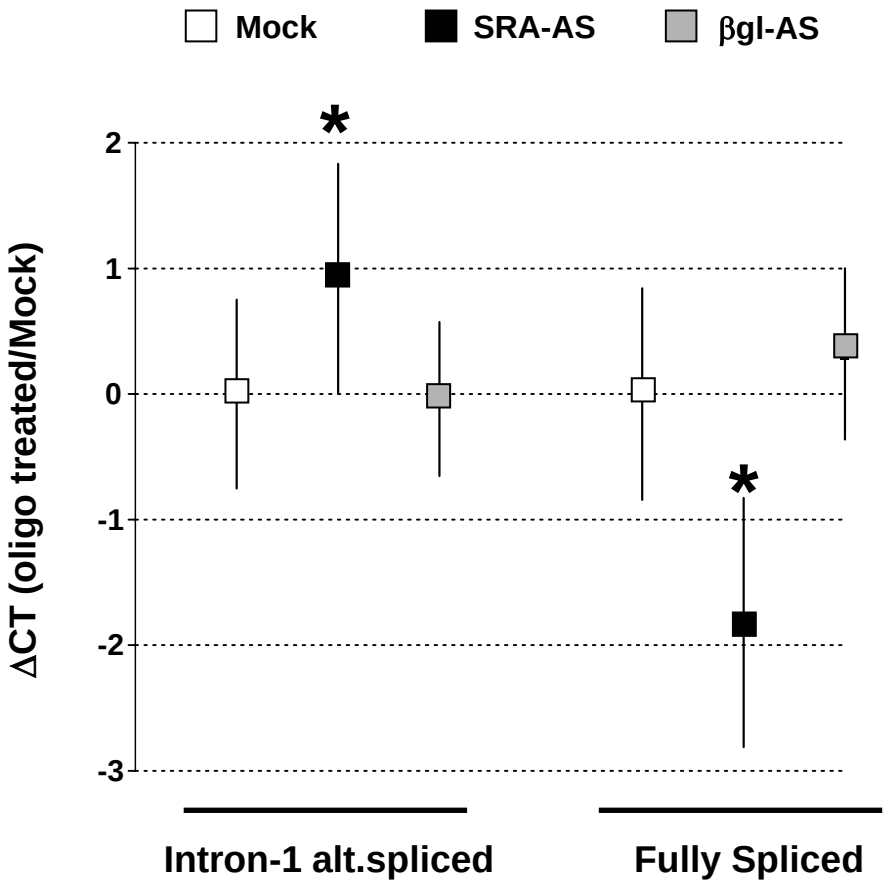
Mock
SRA-AS
βgl-AS

Fig.6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βgl-AS
ΔCT (oligo treated/Mock)
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0
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-3
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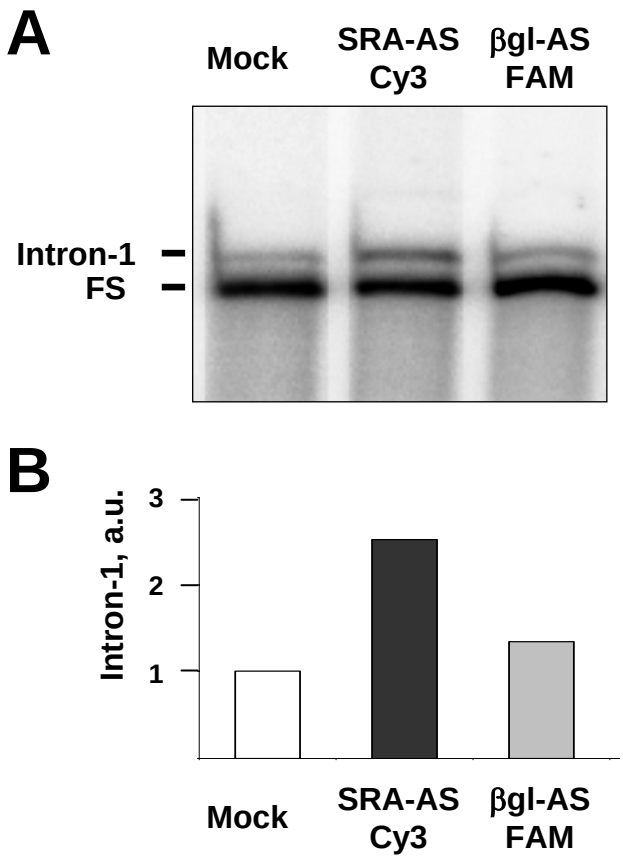
*
Intron-1 alt.spliced
Fully Spliced

Fig.7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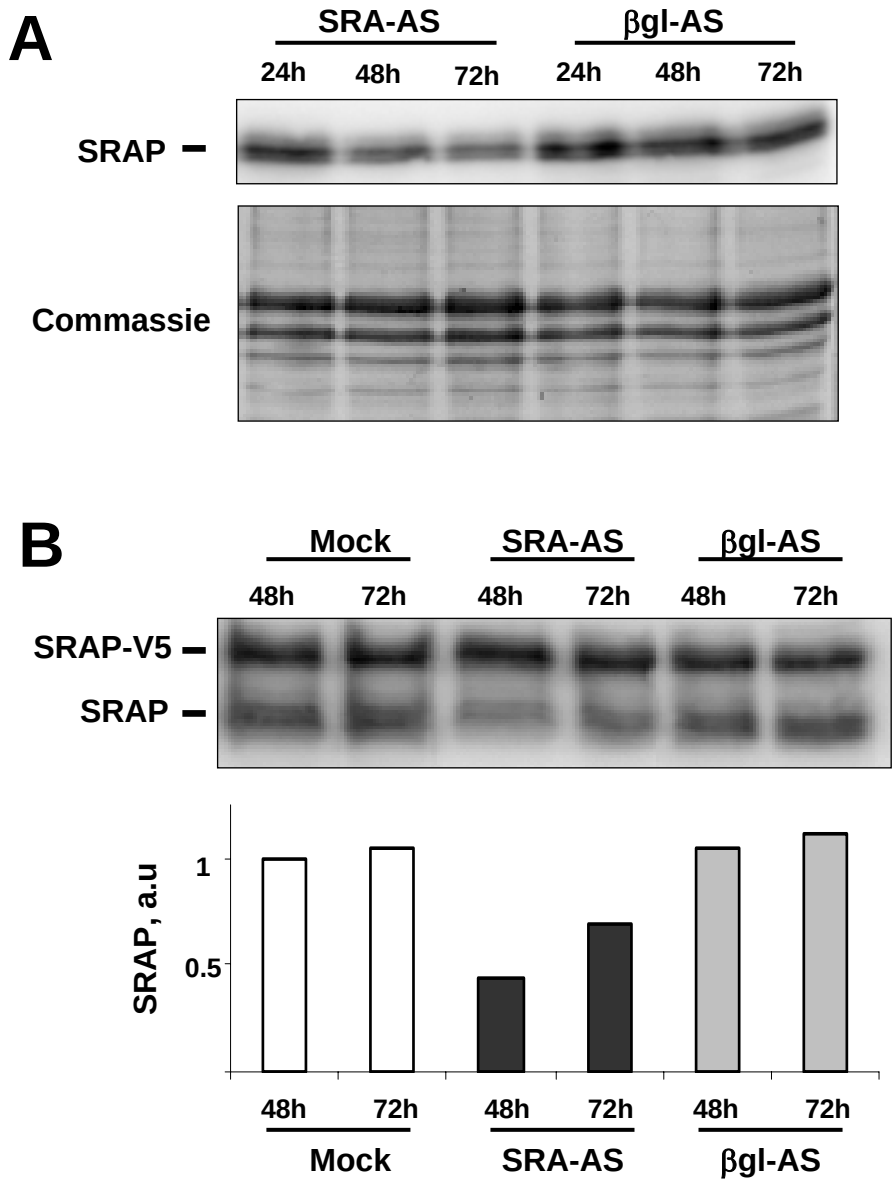
Cy3
βgl-AS
FAM

Fig.8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72h
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72h
SRAP-V5
SRAP
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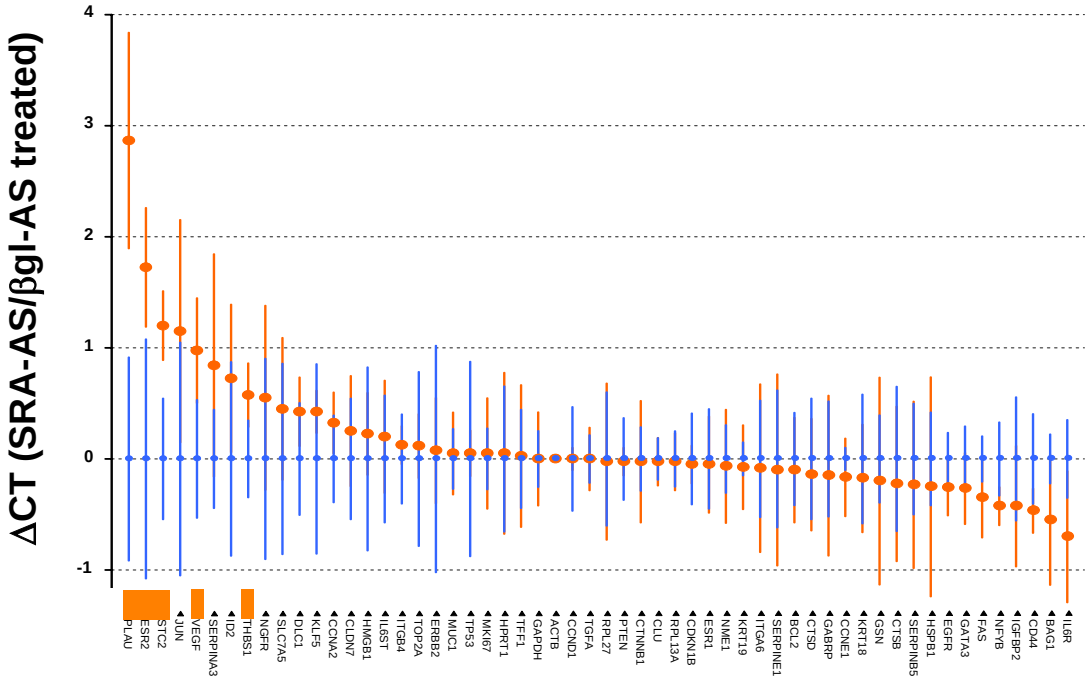
βgl-AS

Fig.9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1
0
-1
PLAU
ESR2
STC2
JUN
VEGF
SERPINA3
ID2
THBS1
NGFR
SLC7A5
DLC1
KLF5
CCNA2
CLDN7
HMGB1
IL6ST
ITGB4
TOP2A
ERBB2
MUC1
TP53
MKI67
HPRT1
TFF1
GAPDH
ACTB
CCND1
TGFA
RPL27
PTEN
CTNNB1
CLU
RPL13A
CDKN1B
ESR1
NME1
KRT19
ITGA6
SERPINE1
BCL2
CTSD
GABRP
CCNE1
KRT18
GSN
CTSB
SERPINB5
HSPB1
EGFR
GATA3
FAS
NFYB
IGFBP2
CD44
BAG1
IL6R

Fig.10

Appendix5:

Change in alternative splicing of the steroid receptor RNA activator (SRA) intron-1 modifies gene expression in T47D5 breast cancer.

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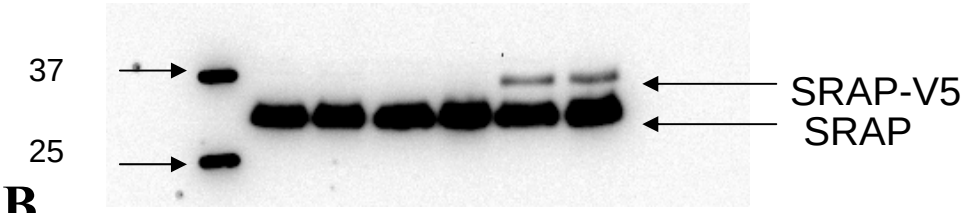
The Steroid receptor co-activator (SRA) has been heavily implicated in estrogen receptor (ER-alpha and ER-beta) signaling pathway. Its expression is altered during breast tumorigenesis and its molecular role in underscoring these events has been suggested. The SRA gene encodes both functional RNA (SRA) and protein (SRAp) products, making it a unique member amongst the growing population of steroid receptor co-regulators. Further adding to the complexity of SRA gene products we have recently reported that a significant number of SRA transcripts have an alternatively spliced intron-1. Retention of this intron forbids the coding of SRA protein product but should not alter the function of the RNA molecule. Interestingly, we have observed varied levels of intron-1 retention within different breast tumor cell lines as well as those derived from different breast tumors.

To investigate the role of SRA intron-1 retention we used splice-switching-oligonucleotides to increase the level of endogenous SRA intron-1 retention in the T47D5 breast tumor cell line. Following experimental optimization, we achieved a fifty-percent conversion in the proportion of non-coding (intron-1 retained) to coding (intron-1 spliced) SRA transcripts. We have observed a persistent effect of this treatment upwards of 72h post-transfection. Using real-time PCR array technologies we found that specific changes in the expression of genes implicated in breast cancer progression and estrogenic signaling are associated with increasing intron-1 retention. Of particular interest is the observed increase in Upa (involved in invasion) and ER-beta (involved in estrogen signaling) gene expressions.

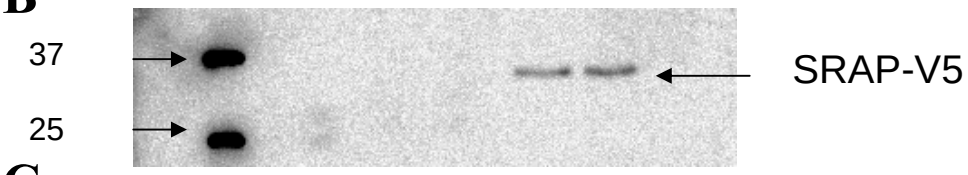
Acknowledgments
This work was supported by grants from CBCRA , CIHR (E.L.), USAMRMC DAMD grants (S. C-K), CCMB, Cancer Care Foundation, TRILINK Research Reward

Appendix6:

A



B

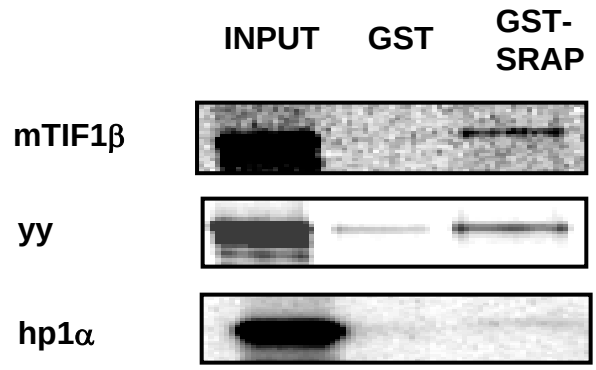


C

lane		
1	M	Marker
2	A	NON-TRANSFECTED T5 cells (negative control)
3	B	T5 cells transfected with pcDNA3.1 V5 His
4	C	T5 cells transfected with pcDNA3.1 Delta ATG V5 His
5	D	T5 cells transfected with pcDNA3.1 DeltaATG SDM1/7 SRA His
6	E	T5 cells transfected with pcDNA3.1 wt SRA1 V5 His
7	F	T5 cells transfected with pcDNA3.1 SDM1/7 V5 His

Figure 1: **relatively low transfection efficiency of different 4 SRA structures in T5 cells.** Different 4 SRA structures shown in the above tableC were transfected into T5 cells with Lipofectamine. The protein lysates after transfection 24 hours were run on a 15% SDS page gel. A) Anti-SRAP antibody (743, polyclonal rabbit antibody from bethyl laboratory1;1000 dilution) was incubated overnight at 4ºC. Secondary anti-rabbit HRP were incubated for 1 hour at room temperature. B) Anti-V5 antibody (1:5000 dilution) was also incubated overnight at 4ºC. Secondary anti-mouse were incubated for 1 hour room temperature.

A



B

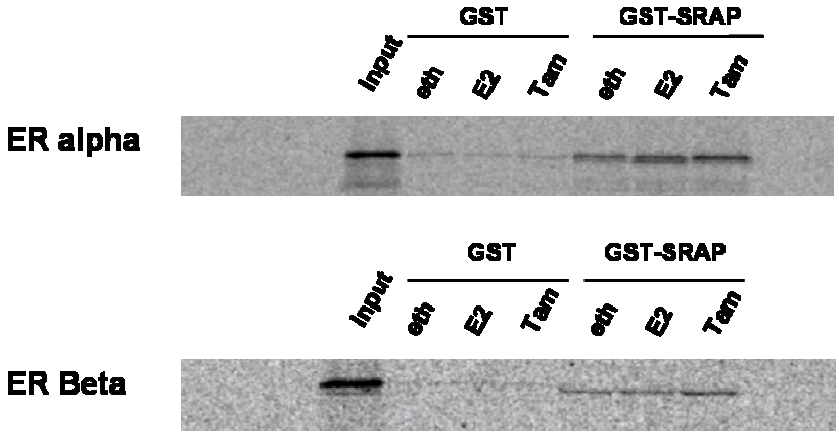


Figure 2: SRAP interacts with ERα, ERβ and mTIF1β YY1, hp1 α in vitro by GST-pull down assay using [S^{35}] –labelled. A) a recombinant purified GST and GST-SRAP were incubated with [S^{35}] –labelled mTIF1β YY1, hp1 α. Following recovery with glutathione agrose, bound protein were resolved on SDA-Page and detected by film. B)ERα, ERβ and purified GST or GST-SRAP sample were treated with ethanol (control), estrodial(10^{-6}M) or tamoxifen(10^{-6}M)